Advances in

VIRUS RESEARCH

VOLUME 7

Advances in VIRUS RESEARCH

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I Introduction

A Purpose and Scope

In the past two decades there have been striking advances in our understanding of the biology of viruses and of the processes involved in their replication These studies, which have thrown considerable light on such basic questions as the chemical nature of the determinants of heredity, and the interrelationships of nucleic acid and protein synthesis, have until recently dealt almost exclusively with bacterial and plant viruses, primarily because these could be studied with much greater precision than was possible with animal viruses During the past few years, however, experimental systems have been developed which allow quantitative chemical, physical, and biological experiments with animal viruses and their host cells Among the major accomplishments were the growth of poliovirus in cultured human (Enders *et al.*, 1949, Scherer *et al*, 1953) and monkey (Syverton *et al*, 1951, Morann and Melnick, 1953) cells, the development of techniques for the growth of single mammalian cells (Puck *et al*, 1956), the development of a plaque assay for animal viruses (Dulbecco, 1952, Dulbecco and Vogt, 1954a), the puri-

fication and crystallization of polioviruses (Schwerdt and Schaffer, 1955, 1956; Schaffer and Schwerdt, 1955, 1959), and the delineation of the nutritional requirements of cultured animal cells suitable for the production of viruses (Eagle, 1955, 1960). With the aid of these techniques, poliovirus has probably been studied in greater detail than any other animal virus, and it is the purpose of this article to consider the recent findings with respect to its biosynthesis. Since some of the techniques, such as rapid purification of the virus, have only recently become available, experiments which will make much of our discussion incomplete are probably being done at the time this is written It is nevertheless hoped that the evidence summarized here will point out the usefulness of poliovirus as a model for studies on the replication of RNA-containing viruses

B Host Cells of Choice

Results of quantitative significance concerning the biosynthesis of poliovirus have come almost exclusively from the use of dispersed mammalian cells, either in primary culture, exemplified by monkey kidney epithelium (Morann and Melnick, 1953), or in serially propagated cultures, such as the HeLa cell (Scherer *et al*, 1953) Either of these cell lines can be used for both the production of virus and its quantitative plaque assay (Dulbecco and Vogt, 1954a; Gifford and Syverton, 1957) Both kinds of cells satisfy most of the requirements for an adequately quantitative system, as suggested by Dulbecco (1955); both permit the measurement of the adsorption rate of virus, the proportion of infected cells, and the virus yield per cell As Dulbecco points out, however, these measurements can best be made with cell suspensions; and this may weigh the balance in favor of the HeLa cell, suspension cultures of which (McLimans *et al.*, 1957) also provide a continuing supply of rapidly growing cells. HeLa cells offer a further advantage in that cultures deriving from a single cell (Puck *et al.*, 1956), and thus presumably uniform in viral susceptibility, can be easily obtained. This consideration assumes importance in light of the wide variations in susceptibility to poliovirus among cultures derived from various primates (Kaplan and Melnick, 1955; Hsiung and Melnick, 1958), and among clonal isolates of the HeLa strain (Darnell and Sawyer, 1959) An additional safeguard against changing cellular susceptibility is provided by the fact that serially propagated cultures can be stored for many months at −70°C. (Scherer and Hoogasian, 1954; Stulberg *et al*, 1958), assuring a reservoir of cells which behave predictably with respect to viral infection

The question as to the host cell with the greatest susceptibility to poliovirus is still unsettled McLaren and Syverton (1957) failed to find

any significant variation in virus susceptibility among monkey kidney cells or a number of parental and clonal human cell types Schwerdt and Fogh (1957) and Fogh (personal communication) have found that primary human amnion cells, or a serially propagated line deriving from human amnion, the FL cell (Fogh and Lund, 1957), gave a higher plaque-titer than any other cell line tested, and five times greater than that obtained with two strains of HeLa cells However, since clonal strains of HeLa themselves differ 10–15-fold (Darnell and Sawyer, 1959), some lines of HeLa cells may be even more susceptible than the FL strain.

Recent reports have suggested that nonprimate cells in culture may acquire the ability to produce poliovirus (Sheffield and Churcher, 1957; Mascoli *et al*, 1958) In one instance this "adaptation" to poliovirus susceptibility was associated with the appearance in the cell of antigens which cross-reacted with antisera to HeLa cells (Melnick and Habel, 1958) This suggests that the culture may have been contaminated with HeLa cells, which thereupon outgrew the original strain Such contamination has been shown to be responsible for at least one instance of the sudden "adaptation" of surviving monkey kidney cells to rapid and sustained growth in cell culture (Simmovitch, personal communication) Another "nonprimate" but polio-susceptible cell strain, originally derived from guinea pig spleen, now has a chromosomal complement similar to HeLa cells (Dulbecco, personal communication), again suggesting the possibility of contamination The one type of nonprimate cell to which poliovirus does definitely "adapt" is the chick embryo fibroblast in primary culture (Roca-Garcia *et al*, 1952, Dunham and Ewing, 1957) While this property could prove useful in the study of the genetic control of host-range variation, the chick fibroblast has no other apparent advantage over either monkey kidney cells or HeLa cells for the production of virus, or for the study of its biosynthesis

C Description of the Virus

1 The Growth Cycle

The initial step of adsorption of poliovirus to a susceptible cell is followed by the phenomenon of "eclipse," the loss of infectivity of the absorbed virus During this time the virus particle presumably enters the cell and is broken down into its protein coat and its infectious component, the nucleic acid (Alexander *et al.*, 1958a,b) Three to four hours after infection (end of eclipse period), new mature virus begins to accumulate intracellularly (Howes and Melnick, 1957; Darnell, 1958) This maturation process is complete within 7–9 hours after infection Spontaneous release of the virus (end of latent period), which begins

about 5 hours after infection, goes on at a much slower average rate than maturation (Howes and Melnick, 1957; Darnell, 1958), and is not completed until 18–24 hours after infection. The production of mature active virus is accompanied by profound chemical changes in the cell (Maassab *et al*, 1957; Ackermann *et al*, 1959; Salzman and Lockart, 1959; Salzman *et al*, 1959), eventuating in its death

2. Purified Virus

a Physical and Chemical Characterization. The pioneer work of Schwerdt and Schaffer (1955, 1956, Schaffer and Schwerdt, 1955, 1959) on the purification of poliovirus has been followed by its physical and chemical characterization. The viral particle is a nucleoprotein sphere approximately 280 A in diameter which crystallizes in an orthorhombic form (Finch and Klug, 1959) The best estimates of its composition show that it is 20–25% ribonucleic acid (RNA), the remainder being protein. Its mass is about 1.1×10^{-17} gm. and its density 1 33 [Dulbecco, Schaffer (personal communications)]. The latter value, determined by sedimentation in a centrifugally established CsCl density gradient (Meselson *et al*, 1957), is lower than the earlier estimates (Schaffer and Schwerdt, 1959) Since the density in CsCl of large molecular weight RNA is approximately 2 0, while that of protein is about 1.29–1 30 (Watson, personal communication), and since the poliovirus particle is 25% RNA and 75% protein, its expected density would be approximately 1 47, rather than 1 33 However, the density of 2 0 for RNA includes the cesium ions which are bound to it. If poliovirus RNA in the intact particle were unable similarly to combine with CsCl because it is already combined, either with a nonexchangeable lighter cation, or with the excess amino groups of the basic amino acids in virus protein, this would account for the apparent discrepancy.

The protein of Type 1 virus contains all the usual amino acids, and no abnormal amino acid (Levintow and Darnell, 1960a) So far as has been determined, the nucleic acid bases are adenine, guanine, cytosine, and uracil The base ratios and percentage compositions are the same for all three antigenic types of poliovirus (Schaffer and Schwerdt, 1959); and since there are no major differences in their biologic behavior in cell cultures, the antigenic types are not distinguished in any of the following discussion. The virus particle contains no deoxyribonucleic acid (DNA), no lipid, and no carbohydrate other than the ribose of the viral RNA (Schwerdt, 1957).

Although the particle appears spherical or polyhedral in ordinary electron micrographs, certain details of structure can be best obtained by the study of virus crystals Finch and Klug (1959) have recently

reported the first X-ray crystallographic studies of poliovirus, which indicate that the particle has an icosahedral configuration made up of sixty identical subunits on the surface. This structure is the same as that of such spherical plant viruses as turnip yellow mosaic (Klug *et al*, 1957a,b) and tomato bushy stunt (Casper, 1956)

According to Crick and Watson (1956, 1957), it should be the protein coat of the virus particle which dictates its icosahedral structure. The postulated sixty protein subunits per poliovirus particle would each have a molecular weight of around 80,000, using Schwerdt's (1957) estimate of 6.7×10^6 as the weight per particle, and a subunit diameter of approximately 60–65 A (Finch and Klug, 1959). It is not known whether this basic unit is divisible into yet smaller units that may or may not be chemically identical, but according to Finch and Klug (1959) this is not ruled out by present evidence.

A relevant question is the number of antigens in the poliovirus particle. Mayer *et al* (1957) have investigated the complement-fixing reactivity of purified virus preparations These purified preparations have two major components, a fraction D which contains virtually all the infectious virus, and a fraction C which appears on the basis of electron micrographs, sucrose density centrifugation, and chemical measurement to contain little or no RNA Although both fractions give equivalent complement fixation with convalescent human serum, fraction D reacts much more weakly than C with the nonspecific antibodies in acute phase serum Finally, the reactivity of D antigen can be changed to that of C by heating, or by large doses of ultraviolet irradiation (LeBouvier, 1957) Fraction C protein may therefore be either a degradation product of poliovirus protein, a precursor, or perhaps part of a subunit which is not exposed as an antigenic stimulus when whole particles are injected However, since crude suspensions and heated preparations are often used to produce antiserum, the conversion of D to C could have taken place prior to immunization, and there may in truth be only one protein in poliovirus This aspect of the problem can now be attacked directly by working with protein obtained from purified virus preparations

b Biologic and Biochemical Behavior. A critically important finding is the fact that the number of characteristic particles visible in the electron microscope is always many times greater than the number of infectious particles (plaque-forming units), the ratio varying between 30 and 2000. This is true for both crude virus suspensions in infected culture fluid, and highly purified material (Schwerdt and Schaffer, 1955, Schwerdt and Fogh, 1957)

The use of radioactively labeled purified poliovirus preparations has provided additional information with respect to its biologic and physio-

chemical behavior Taylor and Graham (1959; Graham, 1959), using virus labeled in its RNA with P^{32} and purified by ion exchange chromatography (Taylor and Graham, 1958), found that the adsorption of radioactivity by monkey kidney cells paralleled the adsorption of plaque-forming activity, indicating that all the particles in their preparations behaved identically with respect to adsorption. Recent experiments in this laboratory, however, using virus purified by cellulose columns and by CsCl density gradient centrifugation (Joklik and Darnell, 1960), indicate the HeLa cells in suspension may adsorb 90–95% of the infectivity, while only 20–30% of the radioactivity remains attached to the cells, whether the isotopic label is in RNA or protein. By adsorbing labeled virus for only five minutes, washing, and then studying the fate of the adsorbed radioactivity, the following points have been established:

1 At both high (1000–2000 particles per cell) and low (1–2 particles per cell) multiplicities, 50–60% of the radioactivity initially attached to the cells is rapidly and spontaneously eluted at 37°C.

2 The specific infectivity (plaque-forming units per count) of the eluted material is very low in comparison with that of the starting virus preparation

3 The eluted fraction does not readsorb.

4 *The eluted virus contains as much extractable infectious RNA as* the starting virus

5 In the nonelutable adsorbed virus, about 50% of the RNA is degraded to acid-soluble material, while most of the other 50% becomes ribonuclease-sensitive Detailed experiments on the fate of this fraction of the viral RNA are in progress

In summary, it would appear that the majority of the particles in a purified preparation of poliovirus attach to the cell surface, and most of these elute spontaneously The fraction of the original virus inoculum that is permanently "adsorbed," as measured by the disappearance of radioactive material from the supernatant fluid, agrees with the fraction that fails to elute from cells which had been exposed to virus for a very short time (5 min.) The discrepancy between these results and those of Taylor and Graham (1959) and Graham (1959) may be due to differences in the method of preparation of pure virus, or in the host cells which were used for adsorption

II Initial Events in Infection

A Adsorption

The initial step in poliovirus infection, as in most other virus infections, is the specific adsorption of the virus to the surface of a susceptible

host cell. Since the development of the accurate plaque assay (Dulbecco and Vogt, 1954a), many factors affecting adsorption rates of infectious virus have been quantitatively explored (The adsorption of noninfectious particles has been discussed in the preceding section, pp 5–6) Bachtold *et al* (1957) found that either calcium or magnesium was necessary for a maximum rate of attachment of virus to monkey kidney cells suspended in a phosphate-buffered saline solution containing NaCl and KCl With optimum concentrations of Ca^{++} or Mg^{++} (10^{-3} *M*) the attachment rate constant was 2.3×10^{-8} ml /min /cell, four times that observed in their absence, and was virtually independent of temperature from 1° to 37°C A slightly slower rate of attachment was observed with suspended HeLa cells in media containing approximately 10^{-3} *M* Ca^{++} and Mg^{++} (Drake, 1958, Darnell and Sawyer, 1960) In contrast to the findings of Bachtold *et al* (1957), Holland and McLaren (1959) reported that Na^{+} facilitated adsorption to HeLa cells to the same degree as Ca^{++} Although they also found no temperature effect in the adsorption of virus to suspended cells, adsorption to monolayers was markedly depressed at 0° compared to 37°C, and was not completely inhibited in the absence of any added cations.

There has been no demonstration that infectious poliovirus, once attached to the cell, can be quantitatively eluted in active form, as can myxoviruses and bacteriophages (Burnet, 1960, Garen and Kozloff, 1959, Garen and Puck, 1951) The attachment of active virus cannot therefore be divided into a reversible and irreversible stage on this basis By analogy with other systems, it is nevertheless probable that initial attachment is a temperature-independent electrostatic phenomenon requiring cations.

B Eclipse and Penetration

Once attachment has taken place, most of the adsorbed virus disappears as infectious material (Howes and Melnick, 1957, Darnell, 1958, McLaren *et al*, 1959), and cannot be recovered by disrupting the cell with sonic vibration, or by freezing and thawing, even though these procedures are known to liberate newly formed intracellular mature virus The small fraction of infectious virus which is recoverable, about 1–5%, is the same whether cells have been singly or multiply infected (Darnell, 1958) At least some of this "cell-associated virus" is insensitive to antiserum (Howes, 1959b, McLaren *et al*, 1959), indicating either that the virus is not held at the cell surface, or that its antigenic site has been masked by its attachment to the cell

The nature of the attachment and eclipse process can now be studied in a cell-free system Holland and McLaren (1959) obtained cell-free

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extracts from sensitive cells (HeLa) which inactivated poliovirus at a rapid rate The activity of this extract was abolished by treatment with trypsin or with ether, but was insensitive to lipase, periodate, or receptor-destroying enzyme from cholera vibrio They suggested that this material may represent a lipoprotein structure from the surface of the cell concerned with viral attachment (It is significant in this connection that nonprimate cells which were resistant to virus infection by virtue of their failure to adsorb virus did not contain the virus-inactivating material)

This thesis is strengthened by the experiments of Quersin-Thiry (1958), who demonstrated that the effect of anticellular serum in preventing infection with poliovirus is due to the fact that adsorption of virus is prevented. Presumably, the antibody reacts with and blocks the adsorptive sites on the cell surface These data all suggest that viral attachment and eclipse may be related phenomena occurring at the cell surface.

The rate at which virus enters HeLa cells, or leaves the immediate surface of the cell, has been measured by several workers (Mandel, 1958; Holland and McLaren, 1959; Darnell and Sawyer, 1960). Cells which had been infected by exposure to virus for only a few minutes were subsequently treated at varying intervals with a potent antiserum. The number of infected cells which were unaffected by this treatment gave a measure of the proportion of cells in which penetration had already occurred For monolayer cells at 37°C, the average time that an adsorbed virus stayed on the cell surface was 30 minutes (Holland and McLaren, 1959) or less (Mandel, 1958), while for suspended cells it was less than 12 minutes (Darnell and Sawyer, 1960). The rate of penetration could be markedly decreased by keeping the cell-virus complex at 22°C. (Mandel, 1958), and penetration was completely prevented at 1°C. (Holland and McLaren, 1959) Holland and McLaren were not able to block the "penetration" reaction with fluoride, azide, dinitrophenol, Tween 80, or cytotoxic cellular antisera Although the entry of virus is temperature-dependent, it apparently occurs independently of at least some types of cellular metabolism.

Once inside the cell, the infecting virus is presumably broken down to protein and nucleic acid, since the viral nucleic acid alone suffices to initiate infection (Alexander *et al*, 1958a,b). (The plaque-forming activity of these nucleic acid preparations is destroyed by ribonuclease, while gamma globulin containing poliovirus antibodies has no effect The converse is true for whole viral particles) The release of the infectious RNA may take place on the surface of the cell, during the process of "penetration," or it could be effected inside the cell; but in any case,

it does not happen sufficiently near the surface to permit added ribonuclease to interfere with infection (Holland and McLaren, 1959).

There is indirect evidence that the breakdown to protein and nucleic acid is effected by the cell, and is not an inherent property of the virus itself. Two strains of HeLa cells which adsorbed whole virus equally well, but which differed 15-fold in the probability of becoming infected, were equally susceptible to infection by infectious RNA (Darnell and Sawyer, 1960). The "resistant" or less susceptible cell was apparently unable to effect the release of RNA from whole virus with the same efficiency as the sensitive cell If this interpretation is correct, the disruption of an incoming virus particle is perhaps a function of the same cellular component which Holland and McLaren (1959) have shown to be capable of "eclipsing" poliovirus in a cell-free system

III Intracellular Events

A Effects on the Cell

Poliovirus infection eventually results in the death of the cell Between the initial stages of infection and that final outcome, however, many changes have been observed Some of these are cytologic, and poorly understood on a chemical basis Others are chemical changes difficult to relate to any process necessary for virus replication, and may be secondary effects in a dead or dying cell which are not directly concerned with virus replication

1 *Cytologic Effects*

Some of the earliest studies on the effects of poliovirus on cultured cells dealt with the microscopic changes occurring during virus production (Ackermann *et al*, 1954, Dunnebacke, 1956a,b, Reissig *et al*, 1956) Althou

picture

human

tures was used in titrating the infectivity of virus suspensions (Enders *et al*, 1949) Although this method is still widely used, especially in large-scale epidemiological work, it has been largely supplanted in quantitative biologic and chemical studies by the elegant plaque assay of Dulbecco (1952)

The first observable microscopic change is in the nucleus, where there is concurrently a central loss of chromatinic material and a peripheral deposition of dark-staining material The nucleus tends to become indented and distorted, and extrudes into the cytoplasm a large acidophilic mass which has been identified as DNA on the basis of its stain-

ing reaction with a fluorescent acridine dye (Tenenbaum, 1957). The nucleolus is discernible throughout the process of infection, and the nuclear membrane also usually remains intact, despite its probably increased permeability (Tenenbaum, 1957)

Although the cytoplasm does not change as dramatically as the nucleus, definite changes have been reported There is a generalized increase in basophilia, most noticeable after the eosinophilic mass has been extruded from the nucleus into the cytoplasm Cytoplasmic vacuoles develop which are reported to move to the edge of the cell (Reissig *et al*, 1956), perhaps concurrently with virus liberation (Lwoff *et al*, 1955) Late in infection, acidophilic masses about 1μ in diameter may appear, which are eventually lost into the medium. During the period of virus release there is hyalinization of the outermost rim of cytoplasm; the surface of the cell can be seen to "bubble," and bits of cytoplasm are pinched off and lost into the medium (Lwoff *et al*, 1955). Tenenbaum (1957) has found that this material contains large amounts of material with the staining characteristics of RNA

As the final reaction, the cells begin to round up, detach from the glass, and float up into the medium. By the time half the cells have detached, 95% of the virus has been released (Dunnebacke, 1956a).

Until recently, the use of the electron microscope in the study of poliovirus-infected cells had not been particularly rewarding. Both Kallman *et al.* (1958) and Harford *et al* (1959) failed to find characteristic intracellular virus particles, despite care to ensure that the majority of the observed cells had actually been infected In possible explanation, Kallman *et al* (1958) have calculated that one would not expect to see poliovirus in electron microscope sections if the particles were distributed randomly, and if there were no more than 10^4 particles within a cell at any given time. However, since the average yield is from 1 to 5×10^3 particles per cell (Schwerdt and Fogh, 1957) and since most of the virus is retained intracellularly for at least several hours after its formation (Howes and Melnick, 1957), viral particles might be expected to be evident even on the basis of these calculations The electron microscope studies have borne out some of the morphologic changes observed with the light microscope, particularly with respect to the development of dense cytoplasmic masses in the infected cell These U-bodies (unknown bodies) were not, however, regarded as virus, but as electron-dense material that had invaded a canalicular system More recently, Stuart and Fogh (1959) have demonstrated in electron micrographs of infected cells cytoplasmic "crystallites" which appear to be comprised of whole virus particles Horne and Nagington (1959), using the phosphotungstic acid negative staining technique of Brenner and Horne

(1959), were able to follow the intracellular development of poliovirus. They concluded that virus protein first appears as subunits, which begin to aggregate about 3 hours after infection Subsequently, typical viral particles with icosahedral structure become demonstrable

One other optical technique, radioautography, promises to contribute materially to the study of poliovirus multiplication. The ready availability of purified virus preparations, and of tritiated nucleotide and amino acid precursors with extremely high specific activity, should make possible some interesting experiments with respect to the fate of an infecting virus particle

2. Metabolic Changes

a Changes in Nucleic Acid and Protein Metabolism. Poliovirus infection is of biochemical interest chiefly because the infected cell soon begins to produce a new nucleoprotein, the virus. Among the major questions that have been posed with respect to the protein and nucleic acid economy of the infected cell, some of which have not yet been clearly resolved, are. (1) does *net* synthesis of macromolecules by the cell continue after infection, (2) does the incorporation of isotopically labeled precursors into cellular macromolecules continue, and if so, (3) does the pattern of incorporation differ from that observed in normal cells?

At first glance, it would seem that the question of net synthesis of macromolecules should be easily answered simply by measuring RNA, DNA and protein in infected cells as compared to uninfected controls Such measurements have been made in several laboratories, but with diametrically opposed results Ackermann and collaborators (Maassab *et al*, 1957, Ackermann, 1958; Ackermann *et al*, 1959) have described net increases in the cytoplasmic RNA and protein of infected HeLa cells, ranging from 100 to 250% of the original values. Since the cytoplasm contained approximately one-half of the cellular protein and RNA prior to infection, their data imply that there is a 50–125% over-all increment in cellular protein and RNA within seven hours after infection It would follow that the infected cell is making new RNA and protein at a rate considerably in excess of the optimum growth rate of normal HeLa cells, which double about once in 16–24 hours What makes these results even more striking is the fact that the experiments were carried out with nongrowing cells, which were not increasing in RNA, DNA, or protein at the time of infection (Ackermann, 1959)

In contrast to these findings, the experiments of Salzman *et al* (1959) show that when rapidly growing suspension cultures are infected, the net synthesis of all macromolecules, whether RNA, DNA, or protein,

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there is no evidence for the breakdown of the latter into small molecular weight material.

b. Miscellaneous Cellular Effects of Poliovirus Infection A number of changes in the intermediary metabolism of infected cells have been reported. Becker *et al* (1958) found an increased uptake of both glucose and PO_4^{---} incident to poliovirus infection in human amnion cells. This was observed early in the infectious cycle, after which the rate of utilization of these substrates decreased to normal, and then subnormal, levels Azide, cyanide, and fluoroacetate had no effect on the yields of infectious virus, but stimulated glucose uptake Levy and Baron (1956, 1957) have made the complementary finding that poliovirus infection in monkey kidney cells stimulated lactic acid production and inhibited the uptake of glycine-C^{14}.

Kovacs (1956a,b) has reported drastic changes in the levels of nucleases and phosphomonoesterases in infected monkey kidney cells prior to the cytopathic effect. However, since all the cells in the culture were not simultaneously infected, it is difficult to relate these changes to a particular stage of virus propagation

Ackermann *et al* (1958) have recorded a toxic effect of cell culture lysates containing poliovirus This effect becomes apparent in monolayer cultures soon after their exposure to the virus-containing medium, well before virus replication has begun, and is evidenced by a less firm adhesion of the cells to glass, so that they are more easily removed on mechanical shaking The toxic factor differs immunologically from virus, and the mechanism of its action is unknown

8 *End Result of Infection*

It has been shown that only cells which give rise to new virus, that is, which have been productively infected, are killed (Darnell and Sawyer, 1960) Relatively "resistant" cells may adsorb virus without becoming infected, and then remain able to grow indefinitely The fate of virus which is adsorbed without producing infection is unknown Resistant cells do not give rise to cultures in which the poliovirus has assumed a "temperate" or "moderate" relationship (Vogt and Dulbecco, 1958). Even normally susceptible cells may continue to grow in the continuing presence of active virus, if there is a proper balance of antiserum, cells, and virus (Ackermann and Kurtz, 1955; Ackermann, 1957) This, however, is simply an expression of the fact that the antiserum protects some of the cells in a culture by preventing infection, while other cells continue to produce virus If the antiserum is removed all the cells become infected and die There is no known counterpart to lysogeny with animal cells and poliovirus

stops abruptly. Rothstein and Manson (1959) also failed to obtain measurable increments in RNA in monolayers of HeLa cells subsequent to infection, and Darnell and Levintow (1960) have confirmed Salzman's findings in relation to protein synthesis. In all these studies, the full cycle of virus production and cell death occurred without any measurable increment in RNA or protein It is clear that an increase in cellular RNA or protein is not a necessary precondition to virus formation, even when the cells were geared to rapid growth at the time of infection The claim of Ackermann *et al.* (1959) that poliovirus has the capacity to "condition certain areas of the cell to synthetic activity" and that "once this condition is induced all templates in the affected area including that of the virus may be expected to function" therefore does not appear tenable

A number of laboratories have followed the incorporation of isotopically labeled precursors into the protein and nucleic acids of infected HeLa cells. Goldfine *et al* (1958) in a careful study, showed that cytidine 2-C^{14} entered the RNA of infected HeLa cell monolayers at 50–75% the rate observed in normal cells, with an even lower rate of uptake into DNA Essentially similar findings were obtained by Salzman *et al* (1959), who showed also that while the RNA uracil was labeled at the same rate in both control and infected cultures, the labeling of RNA cytosine in infected cultures was markedly depressed. This pattern of unbalanced incorporation was, however, not specific for virus infection. Cells which were not growing because of a phenylalanine deficiency similarly incorporated cytidine 2-C^{14} into RNA uracil to a much greater extent than into RNA cytosine.

Maassab *et al* (1957) have obtained quite different results in their studies on P^{32} incorporation into the nucleic acids of the nucleus and cytoplasm Consistent with their finding of a net increase in RNA in infected cultures, they have reported greatly increased P^{32} labeling of RNA in infected cells relative to the controls, and some increase also in the labeling of DNA. In contrast, Rothstein and Manson (1959) found no difference in the rate at which high molecular weight RNA was labeled by P^{32} in poliovirus-infected and normal HeLa cells.

One common finding in the studies of Salzman *et al.* (1959) and Maassab and Ackermann (1959) is that cellular RNA is broken down late in infection, and lost into the medium as nucleosides and nucleotides Salzman *et al.* (1959) have shown that as much as 75% of the RNA of the cell can be lost in this manner, bearing out the cytologic observations that material staining like RNA is lost into the medium (Tenenbaum, 1957). Protein is also lost from the infected cell. However, as Salzman *et al* have pointed out, RNA is lost several hours before protein, and

intracellular pool) at various times after infection permits the determination of the time at which the viral protein and RNA are synthesized, since virus protein or RNA which had already been formed at the time of addition of the label would not be radioactive In the HeLa-S3 cell, viral protein synthesis begins 3 to 3.5 hours after infection, at the same time that mature virus begins to appear (Darnell and Levintow, 1960) Preliminary experiments with labeled adenosine as an RNA precursor indicate that viral RNA also is not formed in any substantial amount prior to virus maturation (Levintow and Darnell, 1960) It thus appears there is a lag of about 2.5–3 hours between infection and the appearance of the virus-specific macromolecules Experiments with P^{32}-labeled virus (Joklik and Darnell, 1960) indicate that only a small fraction of this time is consumed in breaking down the particles to release viral RNA. Moreover, at the time of infection the acid-soluble pool of the cell already contains large amounts of the nucleotide and amino acid precursors of the virus There is at present no explanation for the long period between infection and the synthesis of virus-specific material

It should be emphasized that experiments in which the total viral yield is purified and examined can measure the time at which the bulk of the viral material is synthesized, but do not answer the question as to whether viral RNA or viral protein is the first product in an infected cell LeBrun (1957) has shown that poliovirus antigen can be recognized by the fluorescent antibody technique (Coons and Weller, 1954) in 10% of infected KB cells as early as 1 hour after infection This technique does not, however, permit a quantitative estimate of the amount of antigen formed, and the finding is not necessarily inconsistent with the foregoing studies showing that most of the viral RNA and protein in the final yield were synthesized by the cell between 3 and 6 hours after infection

The exact sites of synthesis and assembly of viral RNA and protein are unknown LeBrun (1957) showed that in a simultaneously infected cell population poliovirus protein first appeared in the cytoplasm, and concluded that the late appearance of antigens in the nucleus probably resulted from damage to the nuclear membrane, permitting the entry of proteins into the nucleus The cytoplasmic structure involved in the synthesis of virus protein remains unknown

Because of the many indications that myxovirus RNA (Liu, 1955a,b, Schafer, 1955, Breitenfeld and Schafer, 1957) as well as cytoplasmic (microsomal) RNA is synthesized in the nucleus (Brachet, 1957, Zalokar, 1958), and because the nucleus is the first morphologically altered cellular structure in poliovirus infection (Ackermann *et al*, 1954, Reissig *et al*, 1956, Dunnebacke, 1956a,b), it is tempting to speculate that polio-

B. Virus Replication

A discussion of the process of poliovirus replication is difficult since so little is known as to the sequence of events between the release of RNA from the infecting virus, and the appearance of mature virus in the infected cell No virus-specific new small molecules or virus-induced enzyme changes, analogous to these found in the T-even bacteriophage-coli system (Wyatt and Cohen, 1952; Flaks and Cohen, 1959; Flaks *et al*, 1959, Zimmerman *et al*, 1959), are known to occur in poliovirus-infected cells Information concerning this most important phase of virus infection derives from three sources. chemical studies with purified virus, genetic studies, and the effects of various chemical and physical agents on infectious virus multiplication.

1 Chemical Aspects of Virus Replication

An absolute requisite for the study of the biochemistry of virus replication is highly purified virus. Methods of cell culture and virus purification have evolved to the point that it is now possible to obtain sufficient quantities of pure virus to study such questions as the source of viral nucleotides and amino acids, and the time course of synthesis of viral RNA and protein Three techniques which have proved particularly useful are the development of methods for the growth of large numbers of poliovirus-susceptible cells in suspension culture under controlled conditions (McLimans *et al.*, 1957), the discovery that treated cellulose columns (Peterson and Sober, 1956; Sober *et al.*, 1956) would adsorb most cellular material, allowing virus to pass through (Hoyer *et al*, 1958, 1959), and the adaptation of cesium chloride density gradient centrifugation (Meselson *et al.*, 1957) to the purification of poliovirus suspensions (Dulbecco, personal communication).

With the aid of these techniques, it has been possible to determine the precursors of viral RNA and protein by differentially labeling, with appropriate isotopic precursors, the cellular macromolecules and the acid-soluble fraction When virus was produced in such cells, the specific activities of its RNA nucleotides (Salzman and Sebring, 1960) and of its protein amino acids (Darnell and Levintow, 1960) were the same as those of the corresponding precursors in the cellular acid-soluble pool, and differed by a factor of 10–20 from the specific activities of the same compounds in the cellular RNA or protein. It follows that poliovirus is formed *de novo* from the cellular pool of amino acids and nucleotides, and not to a measurable degree from breakdown products of the cellular macromolecules

The addition of radioactive precursors to the medium (and thus, to the

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tobacco mosaic virus RNA (Mundry and Gierer, 1958) Poliovirus RNA was inactivated by nitrous oxide exponentially (a one-hit curve), indicating that the integrity of the RNA molecule is necessary for infection. If RNA derived from a virus strain which could not produce plaques under acid agar (d) was exposed to nitrous acid and then allowed to infect cells, the progeny virus contained an increased number of mutants able to produce plaques under acid agar (d+) Since the rate of inactivation was 7000 times that of induced mutation, and since poliovirus contains from 5000 to 6000 nucleotides, he suggested as a likely possibility that deamination in one specific nucleotide produces the mutation from d → d+.

If this interpretation is correct, it implies that, at least under the conditions of these experiments, deamination at any of the many other possible sites in the RNA molecule is usually lethal, and not productive of other observable mutations. It would clearly be of interest to determine the frequency of mutagenesis with respect to another character and, thus, whether this observation holds for a different site in viral RNA.

Another contribution by genetic experiments has been a beginning understanding of the molecular events during the latent period Sprunt *et al.* (1955) first showed that, when HeLa cells were mixedly infected with Types 1 and 2 poliovirus, the progeny virus contained "doubly neutralizable" particles This has since been more clearly and conclusively demonstrated by using plaque-counting techniques, and isolating the yields from single cells (Ledinko, 1959b). These particles are, however, not stable in that they initiate the production of only a single virus type It would therefore appear that a phenotypically mixed Type 1-Type 2 particle contains only one type of RNA, enclosed in some of each kind of protein This suggests the independent synthesis of protein and nucleic acid, and the random "crystallization" of protein subunits around the nucleic acid (cf p 5)

Drake (1958) has studied the "multiplicity reactivation" of poliovirus particles that had been inactivated by ultraviolet light. This phenomenon, first described with bacteriophages (Luria, 1947), involves an interaction between two or more inactive virus particles, or the nucleic acid of these particles, to yield an active unit which initiates productive infection. These experiments can be taken to indicate the existence of a "vegetative pool" in which the naked viral nucleic acid can exchange a damaged part for an undamaged one, with resulting restoration of infectivity Genetic interaction therefore might be expected to occur in mixedly infected cells Although this has not yet been clearly demonstrated, attempts are in progress in several laboratories One difficulty in such recombination studies is that of obtaining stable mutants of

virus RNA also is synthesized in the nucleus. If this were true, it would mean that the two viral components are independently synthesized, and that the viral RNA formed in the nucleus is eventually coated with protein formed in the cytoplasm.

Cytoplasmic microsomal RNA is currently viewed as the governing molecule in protein synthesis (Roberts, 1958). A relevant and central question with respect to poliovirus is whether a single molecule, presumably the infecting viral nucleic acid, can both initiate its own replication and serve as a "template" for the synthesis of viral protein, or whether instead a different RNA molecule is formed which mediates protein formation. The work of Ledinko (1958, 1959a) demonstrates that, in proflavine-treated cells, serologically active protein can be formed and aggregate into spheres without the formation of infectious virus Although it is unknown whether virus RNA was formed under the conditions of these experiments, it does appear that the protein can be formed independently of a final association with newly formed "active" RNA The demonstration by Sprunt *et al.* (1955) and by Ledinko (1959b) that in cells mixedly infected with Types 1 and 2 poliovirus, there is phenotypic mixing in the progeny also speaks for the separate synthesis of RNA and protein.

The elucidation of the relationship between the *de novo* formation of a specific new RNA and of a specific new protein in a virus-infected cell is perhaps the most intriguing present-day aspect of virus research Since both the protein and RNA of poliovirus have identifiable biologic characteristics, the poliovirus-infected cell appears to be an ideal system for exploration in this area

2 *Genetic Aspects of Viral Replication*

Genetic studies have thrown considerable light on the replication of bacterial viruses Such information as the fact that mutant viruses in yields from single cells are clonally distributed (Luria, 1951), that the chance for mating and recombination increases with time (Visconti and Delbrück, 1953), and that recombination is probably related to replication (Levinthal and Visconti, 1953, Levinthal, 1954) aid in understanding the role of DNA in phage production Studies on the genetics of poliovirus have not yet contributed as materially to an understanding of the mode of replication of RNA viruses

A number of experiments with a genetic orientation have, however, provided insight into the process of poliovirus replication Boéye (1959) has recently reported his highly significant experiments with nitrous acid which, presumably by virtue of its ability to deaminate guanine, cytosine, and adenine (Schuster and Schramm, 1958), is a mutagen of

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poliovirus which can be easily scored Among the mutations that have been described for poliovirus are increased plaque size (Dulbecco and Vogt, 1955, Dubes, 1956a), decreased plaque size (Dubes, 1956a; Takemori *et al*, 1957b), inability to incite plaque formation under acid agar (Vogt *et al*, 1957), diminished virulence (Enders *et al.*, 1952; Sabin *et al*, 1954), resistance to bovine serum inhibitor (Takemori *et al*, 1957a, 1958), cystine-requiring, cystine-independent, and cystine-inhibited mutants (Dubes, 1956b; Dubes and Chapin, 1958), relative temperature resistance (Stanley *et al.*, 1956, Dulbecco, 1956), and adaptation to cold (Dubes and Chapin, 1956; Lwoff, 1959). Some of the mutations which would be easiest to score, such as plaque size variation, have proved unreliable because of both the inherent wide variation in the size of poliovirus plaques, and the instability of the mutants.

An interesting observation that relates several of these mutations is the finding that inability to plate under acid agar and inability to plate on a serially propagated line of monkey kidney cells are both associated with avirulence for monkeys (Vogt *et al.*, 1957; Kanda and Melnick, (1959)

The studies of Vogt *et al* (1957) and Dulbecco and Vogt (1958a,b) have perhaps contributed most to the present understanding of the genetics of virus replication. The mutation rate of poliovirus with respect to the ability to initiate plaques under acid agar was found to be of the order of $2\text{–}3 \times 10^{-5}$ mutations per particle per duplication They were also able to demonstrate that some strains reverted to the ability to plate under acid agar in one step, while others took several steps, indicating several mutational sites for a single character. One of the most convincing pieces of evidence for the existence of a "vegetative" pool of multiplying RNA during the latent period also comes from the work of Dulbecco and Vogt (1958b), who found that proflavine exerted a mutagenic effect on poliovirus if supplied to the cell during the latent period, but had no effect either on the infected cell subsequent to maturation, or on the virus itself

3. *Implications of the Effect of Various Chemical and Physical Agents on Virus Formation*

There have been a number of studies on the effect of some physical or chemical variable on poliovirus production. One of the important questions which has been approached in this manner concerns the role of DNA in the production of the RNA-containing poliovirus Fluorouracil and fluorodeoxyuridine are inhibitors of thymidine synthesis (Heidelberger *et al*, 1957, Cohen *et al*, 1958) With these compounds, Simon (1959) and Salzman (unpublished experiments) have shown that

amounts which completely inhibit DNA synthesis in HeLa cells fail to reduce poliovirus yields. Simon has also shown that when as much as 85% of the thymine of DNA is replaced by an analog, 5-bromuracil, the HeLa cell is still capable of producing normal amounts of poliovirus This indicates not only that new DNA synthesis is unnecessary for poliovirus production, but that the structure of a large portion of the cellular DNA can be altered without affecting the ability of the cell to synthesize a specific viral RNA and protein Salzman (unpublished experiments) has made another interesting observation with 5-fluorodeoxyuridine. Although this compound is primarily an inhibitor of DNA synthesis, at sufficiently high concentrations it will also inhibit net RNA synthesis Even under these circumstances, however, incorporation of radioactive precursors into RNA ("RNA turnover") continues, and if such cells are infected with poliovirus they produce full yields It is not clear whether the capacity of the fluorodeoxyuridine-inhibited cell to make poliovirus means (a) that cellular RNA turnover provides a mechanism for virus synthesis, (b) that viral RNA is made preferentially from the pre-existing normal ribonucleotide pool, or (c) that the analog is itself incorporated into virus RNA, as it apparently can be without affecting the infectivity of tobacco mosaic virus RNA (Gordon and Staehlin, 1958)

There have been a number of studies on the nutritional requirements for poliovirus synthesis by the cell Early observations that both the HeLa (Eagle and Habel, 1956, Darnell and Eagle, 1958) and monkey kidney (Rappoport, 1956) cell could make normal amounts of virus in a medium lacking most of the amino acids and all of the vitamins required for cellular growth pointed to the cell itself as the source of the amino acids and nucleotides necessary for virus synthesis Later experiments showed that, under conditions in which the cellular pool of free amino acids was depleted, the growth-essential amino acids became necessary for virus synthesis (Darnell *et al*, 1959) The importance of the acid-soluble pool in supplying the precursors for virus synthesis has now been proved by studies with isotopically labeled virus (Darnell and Levintow, 1960)

The large supply of preformed compounds in the pools of infected cells may account for the fact that blocking glucose utilization, either with inhibitors (Becker *et al*, 1958) or by anaerobiasis (Gifford and Syverton, 1957) seems to have no effect on poliovirus production In addition, the well-known ability of cells in culture, particularly HeLa cells, to metabolize substrates anaerobically may provide a further source of "high energy" compounds used in virus synthesis, in the absence of aerobic metabolism

poliovirus which can be easily scored Among the mutations that have been described for poliovirus are increased plaque size (Dulbecco and Vogt, 1955, Dubes, 1956a), decreased plaque size (Dubes, 1956a; Takemori *et al*, 1957b), inability to incite plaque formation under acid agar (*Vogt et al*, *1957*), *diminished virulence* (*Enders et al.*, *1952; Sabin et al*, 1954), resistance to bovine serum inhibitor (Takemori *et al*, 1957a, 1958), cystine-requiring, cystine-independent, and cystine-inhibited mutants (Dubes, 1956b; Dubes and Chapin, 1958), relative temperature resistance (Stanley *et al*, 1956; Dulbecco, 1956), and adaptation to cold (Dubes and Chapin, 1956; Lwoff, 1959). Some of the mutations which would be easiest to score, such as plaque size variation, have proved unreliable because of both the inherent wide variation in the size of poliovirus plaques, and the instability of the mutants.

An interesting observation that relates several of these mutations is the finding that inability to plate under acid agar and inability to plate on a serially propagated line of monkey kidney cells are both associated with avirulence for monkeys (Vogt *et al.*, 1957; Kanda and Melnick, (1959)

The studies of Vogt *et al* (1957) and Dulbecco and Vogt (1958a,b) have perhaps contributed most to the present understanding of the genetics of virus replication The mutation rate of poliovirus with respect to the ability to initiate plaques under acid agar was found to be of the order of $2\text{–}3 \times 10^{-5}$ mutations per particle per duplication They were also able to demonstrate that some strains reverted to the ability to plate under acid agar in one step, while others took several steps, indicating several mutational sites for a single character. One of the most convincing pieces of evidence for the existence of a "vegetative" pool of multiplying RNA during the latent period also comes from the work of Dulbecco and Vogt (1958b), who found that proflavine exerted a mutagenic effect on poliovirus if supplied to the cell during the latent period, but had no effect either on the infected cell subsequent to maturation, or on the virus itself

3. Implications of the Effect of Various Chemical and Physical Agents on Virus Formation

There have been a number of studies on the effect of some physical or chemical variable on poliovirus production. One of the important questions which has been approached in this manner concerns the role of DNA in the production of the RNA-containing poliovirus Fluorouracil and fluorodeoxyuridine are inhibitors of thymidine synthesis (Heidelberger *et al*, 1957; Cohen *et al*, 1958). With these compounds, Simon (1959) and Salzman (unpublished experiments) have shown that

C. Maturation

The final intracellular event in poliovirus formation by an infected cell is maturation—the union of virus RNA and protein. The insensitivity of whole virus to ribonuclease (Alexander *et al*, 1958b), and the X-ray crystallographic evidence of identical protein subunits (Finch and Klug, 1959), makes it probable that maturation entails the "crystallization" of the protein around the RNA The nature of the bonding between the nucleic acid and the protein is unknown. The results of the amino acid analysis of the virus protein are, however, provocative in this connection (Levintow and Darnell, 1960a). The poliovirus RNA contains approximately 6000 nucleotides, and the total protein contains approximately 6000 basic amino acid residues which could serve to bind the RNA

The time of onset of maturation has been shown to be asynchronous to some degree in individual cells, some beginning to accumulate new virus as much as 2–3 hours in advance of others. Howes' (1959a,b) data with single cells indicate, however, that 80–90% of HeLa cells have reached maximal yields by 7 2 to 8.2 hours after infection This is in agreement with the maturation curves as determined in mass cultures (Howes and Melnick, 1957, Darnell, 1958). One factor that affects the onset of maturation is the multiplicity of infection. Multiply-infected cells begin producing intracellular virus about 1 hour earlier than singly infected cells An adequate explanation for this observation is lacking

The maturation process proceeds until virtually all the virus protein has been formed into particles. This was demonstrated by Mayer *et al* (1957), who showed that virtually all the material in infected tissue culture fluid which was serologically active as poliovirus could be recovered in the purified virus particles Whether all the virus RNA produced by the cell is similarly incorporated into particles is unknown

IV. Virus Liberation

The study of Lwoff *et al.* (1955) on the release of poliovirus from single infected monkey kidney cells indicated that virus release occurred in a "burst" approximately 6 hours after infection In this respect single cells differ from mass cultures, in which virus production is complete 7–9 hours after infection, but in which virus release is not complete for 12–18 hours This is true of both monkey kidney (Howes and Melnick, 1957) and HeLa (Darnell, 1958) cells, and of both suspended cell cultures and monolayers Howes (1959b) has shown that the lag between the production and release of virus in both monkey kidney and HeLa cell cultures is due to an asynchrony in release time, but that once a

These findings with poliovirus contrast with the results obtained with a number of viruses grown in the chorioallantoic membranes of embryonated chicken eggs (Ackermann, 1951), and with Western equine virus grown in cultured chicken embryo cells (Dulbecco and Vogt, 1954b), all of which required actively metabolizing cells for virus production. As Gifford and Syverton (1957) point out, the difference is possibly in the host cells, rather than in the mechanism of viral replication.

Variation in temperature during the latent period exerts a profound effect on the poliovirus-infected cell, in terms of the output of infectious virus, and genetic variations with respect to this temperature effect have been described Dubes and Chapin (1956) showed that a strain of virus which was adapted to growth at 30° failed to multiply at 36°C. Conversely, the parent 36° strains grew poorly at 30°C. in comparison with the mutant Lwoff has elaborated on these findings, and demonstrated a range of poliovirus strains with a wide spectrum of critical temperature optima (Lwoff and Lwoff, 1958, Lwoff, 1959; Lwoff and Lwoff, 1959). With one strain of Type 1, an increase in temperature from 37° to 40°C. decreased the viral yields by more than 99%. If the cultures were returned to 37°C. within 3 hours after infection the cells produced virus almost as well as controls, with respect to both the time of production and the amounts of virus produced. Virus production could, however, be interrupted by raising the temperature at any time during the period of rapid increase in intracellular virus This indicates that the higher temperature does not affect events during the early latent period, but inhibits some reaction(s) late in the "vegetative phase" Since it is now known that this is the time when both viral protein and RNA are being formed (Darnell and Levintow, 1960; Levintow and Darnell, 1960b), it seems a good possibility that one or both these molecules is formed improperly, or in insufficient amounts, at adverse temperatures

The inhibitory effect of pH fluctuations in the medium on poliovirus formation was first observed by Gifford *et al* (1956) They related this to cellular respiration, and were able to show that at pH levels at which the production of virus was inhibited, oxygen uptake and glucose utilization were also diminished Vogt *et al* (1957) have since shown that some virus particles can apparently cause plaque formation under acid agar, while other mutant forms either cannot, or do so very slowly They failed to find a difference in the growth rates of these two strains of virus in cell suspensions at pH 6 8 and 7 4 Lwoff (1959) has extended these observations to show that pH 6 9 and below is generally inhibitory for most virus strains, and that pH 6 6 reduces the viral yield to less than 0 1% of controls.

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cell does start to release its virus it usually does so within an hour, in agreement with Lwoff *et al* (1955). He showed also that, while some cells in infected cultures liberate virus as early as 6 hours, for most of the cells this occurs later.

The chemical or physical mechanism for virus release is unknown. It *appears to be liberated free of any attached cellular material*, as indicated by the absence of cellular antigens in purified virus preparations (Levintow and Darnell, 1960a).

V. Conclusion

It becomes increasingly clear that the brilliant studies with bacteriophage and tobacco mosaic virus represent an invaluable legacy to the investigator interested in animal viruses. Many of the most illuminating experiments with poliovirus are restatements of problems already answered for these two systems The particular value of poliovirus is that it is an RNA-containing virus, the replication of which can be studied under experimental conditions approximating those of the coliphage system It may not be overly optimistic to anticipate in the near future experiments with poliovirus modeled after such classic bacteriophage experiments as virus "suicide" by incorporated P^{32}, genetic recombination (*perhaps with nucleic acid alone*), and the study of molecular transfer from parent to progeny.

The detailed study of the chemical and biophysical properties of the virus particle promises to be rewarding in itself. The ultimate size of the subunit of poliovirus protein is an important and unanswered question. It is a particularly challenging problem, since the genetics of poliovirus may develop in the next few years to the point that it would become possible to correlate genetic changes in the RNA with possible chemical changes in the protein coat There is preliminary evidence for free sulfhydryl groups in the protein coat of poliovirus (Strohl, 1958). This should allow attachment of heavy atoms such as mercury to yield a product which would be of aid to the X-ray crystallographer in studies on the structure of the particles.

The prospects for rewarding exploration with poliovirus are bright. A reappraisal several years hence of the process of poliovirus biosynthesis should be interesting reading indeed.

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THE RELATIONSHIPS OF THE VIRUSES OF MEASLES, CANINE DISTEMPER, AND RINDERPEST

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I Introduction

The advent of simplified cell culture techniques has accelerated investigation of several comparatively neglected diseases of man and animals Prominent among those infectious agents on which knowledge is accumulating at a rapid rate are the viruses of measles, canine distemper, and rinderpest A renewed interest in measles has arisen because of the relative ease with which this agent can now be grown in the laboratory, the continuing importance of measles infection in man, and the bright outlook for its control through the development of effective vaccines In addition, the older speculations of viral association have been recently supported by experimental observations of a relationship between measles and canine distemper viruses and between distemper and rinderpest All of this has led to considerable work in several widely

separated parts of the world aimed at answering many intriguing questions Do these three viruses, of man, canines, and cattle, represent strains of a common family? Do they cause attenuated infection in other than their natural hosts and does a sufficient degree of cross-protection result to be of value in the development of vaccines?

The material in this section provides essentially a continuation of the chapter "Measles Virus" by Black *et al.*, in Volume VI of this series (1959). More recent publications provide several examples of the variability in behavior of a given virus which can appear with differences in cell substrate, nutrients, virulence level of the strain, or its passage history. The range of mutability of virulence is wide in this "family" (the quotation marks are required at present), suggesting that systematic investigation of strain attenuation or host adaptation along genetic lines might prove worthwhile.

II. Measles Virus

For a review of the earlier literature see the article on "Measles Virus," by G Rake, in *Viral and Rickettsial Infections of Man* (T. M. Rivers and F. L. Horsfall, Jr., eds), Lippincott, 1959.

A. Cultivation

1 Mammalian Cell Cultures

The spectrum of cell types capable of supporting the growth of measles virus has continued to widen, but there appear to be no significant advantages in the use of a particular human cell. Dog kidney tissue is reputed to be of maximal sensitivity and has been found to be of value for the isolation of virus from clinical material Its relative freedom from extraneous agents other than canine hepatitis and its longevity in cultures commend it for this purpose (Frankel *et al*, 1958). The report of Wright (1957) that measles virus grown in rhesus kidney tissue could produce cytopathic effects in mouse, hamster, and guinea pig kidney cultures has been partially confirmed in several laboratories by the successful serial propagation of measles in suckling mice and hamsters (see below), indicating a ready potentiality for adaptation to rodent tissues However, no report has yet appeared of the actual growth of measles in rodent cell cultures Using bovine embryonic tissues, Girardi *et al.* (1958) were unable to obtain measles cytopathic effects in cultures of muscle, lung, heart, or kidney through three passages each of which was observed for 35 to 45 days. Complement-fixing antigen was absent from the harvest fluids More recently Schwarz and Zirbel (1959) have successfully established a strain of measles virus in bovine embryonic

kidney tissues with typical giant cell CPE They present suggestive evidence of its rapid attenuation for cynomolgus monkeys after only a few bovine cell transfers Although a wide variety of conventional media formulations have been found suitable for growth of measles virus, any specific nutritional requirements remain unknown

Measles virus produces plaques characterized by small size, clear centers, and sharp margins in kidney monolayers from several species of monkeys (Hsiung *et al*, 1958, Black, 1959a) and in HeLa cells (Underwood, 1959). By means of plaque assay under a variety of conditions, it has been shown that measles has an attachment time of 3 to 4 hours, considerable infectious virus remains within the cell throughout the fairly long life of most cultures Infectious supernatants can be harvested and replaced repeatedly with fresh medium in the system until all cells are eventually destroyed Measles is a thermolabile virus and titers in cultures maintained at 32°–33 5°C often exceed those in cultures grown at 37°C Black (1959a) has noted a constancy of virus titer which is the result of an equilibrium between virus release and decay at temperatures around 37°C, with a half-life of approximately 2 hours at this temperature In contrast, pH stability is high, and no change in infectivity is observed in preparations maintained for 3 hours at pH levels between 6 0 and 10 5

Cytopathic effects and complement-fixing (CF) antigen in measles-infected mammalian cell cultures usually become apparent at about the same time but rarely are detectable before the third and usually by the fifth day after inoculation In our own experience, using measles virus grown in human heart cell cultures, complement-fixing activity was not detectable in undiluted fluids until titers of the order of 10^5 °$TCID_{50}$/ml were reached However, infective titer is not always a reliable guide to antigen level, especially in older cultures, because of the thermolability of measles virus Furthermore, as shown by Black (1959a) and by Rapp and Gordon (1958), considerable CF antigen remains within the cells of a culture until released by mechanical rupture or by the cytolysis of infection Since virus accumulates both intra- and extracellularly, where measles antigen is an objective of culture it may prove desirable to use a low multiplicity of virus for initiating infection so as to prolong the life of the system over several weeks Data along these lines have been provided by Underwood (1959).

Although definitive information is not available, the labile nature of measles virus is apparent even at the subzero temperatures customarily used for virus storage We have observed a drop of a thousandfold in virus fluids stored at −15 to −20°C for 60 days and Girardi *et al* (1958) report a loss of 2 8 $TCID_{50}$/ml in material stored for 18 weeks

at $-70°C$. Although freeze-drying is attended with some initial loss of infectivity, fresh growth could be readily initiated in our laboratory from measles cultures lyophilized with 5% added rabbit serum after 2 years of storage at $+5°C$. A variety of proteins including bovine and human serum albumin, gelatin, milk and peptone broth will decrease the rate of spontaneous inactivation of the virus in fluids kept at $+5°C$.

2. *Embryonated Eggs*

Older investigations of the growth of measles in embryonated egg membranes, while establishing the basic observation that this virus could be propagated in certain avian cells, were hampered by the lack of a simple index of viral multiplication (Enders, 1940; Rake and Shaffer, 1940). The appearance of clinical signs in experimentally infected monkeys or human volunteers was the only marker available, and the value of this was diminished by the high susceptibility to measles infection among primates in captivity. However, the availability of *in vitro* indicator systems has led to a profitable series of reinvestigations of measles growth using both embryonated eggs and chick cell cultures (Enders *et al*, 1957; Milanovic *et al.*, 1957; Katz *et al.*, 1958a; Gordon *et al.*, 1959).

Direct inoculation of eggs with measles strains from man or in early tissue culture passages (2 or 3) failed to yield an agent when human cell cultures were employed as the indicator system. However, during the course of serial passage in human amnion cells, the Enders group, to whom we owe much of our information, observed a spontaneous alteration in the cytopathic effects of the agent which was associated with its ability to propagate in the chick embryo. Although human amnion cultures of early passage virus contained numerous multinucleate "giant" cells, at about the fourteenth transfer an altered response occurred consisting of the appearance of stellate, fusiform, fibroblast-like cells When the twenty-eighth passage of this particular (Edmonston) strain was placed within the amniotic sac of chick embryos, proliferation of measles occurred The eggs did not die but multiplication was demonstrable by the production of cytopathic effects in human cell tissue cultures, dilution assays, and identity neutralization tests.

The importance of avian amniotic tissues as a major locus of infection is stressed by several workers However, successful transfer in eggs, even via the yolk sac or chorioallantoic space, is possible if amniotic membranes or fluid are used for passage material. Average infectivity titers of the Edmonston strain in egg material have been of the order of $10^{4.0}TCD_{50}/ml$, but lethal effects are rare Although CF antigen has been low or absent from crude infectious tissue suspensions, Taniguchi *et al.* (1956), using a Japanese strain of measles, obta' ple-

ment-fixing activity in infected egg tissues after the suspension was given three cycles of concentration at 22,500 r p m in the ultracentrifuge

3. *Avian Cell Culture*

Adaptation of measles to eggs led to a reinvestigation of its behavior in cultures of chick embryonic tissues. Milanovic *et al* (1957) and Katz *et al* (1958a) studied virus which had 24 serial passages in human kidney cells, 28 in human amnion cultures, and 6 in embryonated eggs. In cultures prepared as monolayers from trypsinized whole embryo, the agent multiplied without cytopathic effect for 4 transfers Growth was slow, reaching maximal titers between the twentieth and fortieth day. During the fifth passage, a change appeared reminiscent of that occurring with human cell and egg passage material This was characterized by a more rapid growth of the virus and the appearance of typical fusiform alterations of the cells Maximal titers between $10^{4.5}$ and $10^{5.8}$ TCD_{50}/ml were regularly obtained by Katz *et al* (1958a) Strains of measles adapted to avian cells readily infect primate or human cell cultures but, as is the case of infected chick embryo materials, produce less CF antigen for reasons still obscure

4 *Growth in Rodents*

By means of intracerebral inoculation of the Edmonston strain from HeLa cell cultures (Imagawa and Adams, 1958), or a strain (Krech) passaged in human kidney cells (Carlstrom, 1959a) a fatal, transmissible, and infectious disease could be produced in newborn mice Symptoms appeared on the fifth to tenth days and consisted of spastic paralysis and/or lethargy This was indistinguishable from that caused by the inoculation of canine distemper virus Death invariably occurred one to two days after the onset of signs With an Edmonston derivative of virus, levels in brain tissue averaged $10^{3.8}$ LD_{50}/ml in early passages and were not changed appreciably after 85 serial intracerebral transfers (Adams, 1959). Identity of each of the mouse-adapted strains has been adequately checked by neutralization tests

In suckling hamsters Burnstein *et al* (1958) were able also to establish a line of measles virus by intracerebral inoculation Although a fatal meningoencephalitis occurred, resembling that produced in mice, complement-fixing antigen could not be detected in tissues of the central nervous system (CNS).

B. *Attenuation of Measles Virus for Primates and Man*

The discovery that measles virus, after passage in certain systems, becomes attenuated for monkeys and man promises a method for the control of this still formidable epidemic disease by means of active

immunization Several approaches to this problem had been reported even prior to the growth of measles in tissue culture. In the U S S.R, attempts were made to vaccinate children with live measles virus from the blood of infected puppies as early as 1953. Unfortunately, the available reports on these studies are only fragmentary and few English translations exist It was claimed that while disease was observed in all recipients of this "attenuated" measles strain, it was of a mild nature and complications were less frequent than in a control group (Ioffe, 1953) In Japan, Arakawa (1954), after prolonged serial passage, prepared a live measles vaccine from brain suspensions of infected mice. Protection against natural infection was reported, and recipients developed specific antimeasles neutralizing antibody. Again it is difficult to assess the significance of this work in the absence of more detailed references.

Adaptation of virus to chick embryo or chick embryo tissue culture has been the method most widely employed for attenuation. This follows the course attempted by Rake and Shaffer (1940) but abandoned by them in the face of the difficulties of laboratory diagnosis The earliest of recent papers again comes from the U.S S R (Zhdanov and Fadayeva, 1956) where strains, which had been isolated in human lung culture and passed in this system for from 8 to 50 transfers, were then inoculated simultaneously into the amnion, allantois, and yolk sac of 9-day chick embryos After 3 days of incubation at 36°C., the allantoic fluids were harvested and lyophilized The infectious titer of the dried measles preparation is not stated A preliminary study was performed in 214 children, using vaccine from avian passage and administered intranasally as a 1:10 dilution of the dried allantoic fluid. Although a symptom complex characteristic of typical measles was not seen in any of the group, a variety of mild findings was noted Excretion of virus from the nasopharyngeal tract or viremia was found in 26%, leukopenia in 14%, a temperature greater than 37°C. in 9%, and a rash in 4% of the children. Koplik's spots were not recorded, although "upper respiratory catarrh" was noted in 4% of the group Rather surprisingly, CF antibodies were found only in 48% after three doses of vaccine. These were measured against an antigen prepared from measles-infected allantoic fluid, a source from which most workers have been unable to obtain satisfactory complement-fixing reagents In a subsequent study, using virus of later passage number, a total of 2643 Russian children was reported as uneventfully vaccinated with the attenuated measles material Information as to the degree of protection conferred by these trials is not available, and a lack of critical quantitative data makes it difficult to evaluate these findings Nevertheless, one must recognize

the originality and enterprise involved in the intranasal inoculation of children with a chick embryo adapted measles variant.

The systematic exploration of measles etiology by the Enders' laboratory has included a considerable emphasis on several facets of attenuation (Enders *et al*, 1959a, 1959b; Katz *et al*, 1958b). When susceptible *Macaca* monkeys, i e, devoid of measles antibody, were inoculated with measles virus derived from early passage human kidney tissue culture, a viremia developed whether infection was by the intravenous, intranasal, or intracerebral pathways Although a moderate leukopenia and a rash were observed in half of the animals, CF antibody invariably appeared in all at about the second week In contrast, when the same strain of measles virus had been carried through a further series of transfers, first in chick embryo and then in chick embryo tissue culture, it was no longer capable of inducing any clinical signs in small groups of monkeys, even when injected intravenously or into the central nervous system [see Table I (from Enders, 1959a)] Virus was not detectable in the blood stream, throat washings, or cerebrospinal fluid That infection actually occurred was evident by the regular appearance of antibody but after a slightly longer interval than was observed in the case of monkeys infected with nonattenuated measles virus Titers were equal to those found in animals convalescent from infection with the virulent strain

TABLE I

TESTS FOR VIRUS, NEUTRALIZING, AND COMPLEMENT-FIXING ANTIBODIES IN 2 MONKEYS INOCULATED INTRACEREBRALLY AND INTRACISTERNALLY WITH "VIRULENT" AND "CHICK CELL" VIRUS, RESPECTIVELY[b]

Day after inoculation	"Virulent" virus[a]					"Chick cell" virus[a]				
	Bl	TS	CSF	NT	CF	Bl	TS	CSF	NT	CF
0	0	0	0	<4	<4	0	0	0	<4	<4
1	0	+	—	—	<4	0	0	—	—	<4
3	+	+	+	—	<4	0	0	0	—	<4
5	+	+	—	—	<4	0	0	—	—	<4
7	+	+	+	—	<4	0	0	0	—	<4
9	0	+	—	—	<4	0	0	—	—	<4
11	0	0	0	—	16	0	0	0	—	<4
13	0	0	—	—	64	0	0	—	—	256
17	0	0	0	1,400	512	0	0	0	840	1,024

[a] 0, no virus isolated, +, virus isolated, —, no test Bl, blood, TS, throat swabbing; CSF, cerebrospinal fluid, NT, neutralizing antibody titer of serum, CF, complement-fixing titer of serum

[b] From Enders *et al*, 1959a.

When monkeys convalescent from attenuated measles were re-exposed after 175 days to virulent, early passage measles by means of intravenous and intranasal inoculation, they remained healthy and no circulating virus was found. A highly significant observation was the recovery of virus from the throats of these immune monkeys, suggesting that local, restricted proliferation of the agent occurs in the immunized host.

Several important questions can be formulated around these observations. To what extent does virulence for *Macacus* monkeys parallel attenuation for man? Would a host immunized with attenuated measles constitute a reservoir of "natural" measles if the latter were to multiply in a nonsymptomatic fashion in the respiratory tract? What is the likelihood that reversion of "attenuated" measles to a more severe clinical form can occur if an occasional individual receiving live measles vaccine is able to shed virus and infect fully susceptible contacts? Have attenuated strains the capacity to cause encephalitis? Partial answers to some of these problems are now at hand; others should be forthcoming in the near future.

Vaccination of susceptible, antibody-free children with the avian-type, attenuated, Edmonston strain of measles was begun in 1957 by Enders and associates (Katz and Enders, 1959) and continued by Kempe and Maisel (1959) using the same material. While it is premature to present a quantitative review of the available results, these investigations appear to have progressed to the point where it can be stated with some assurance that avian cell passage virus is attenuated in its virulence for man This is based upon the difference between the mild disease it induces and epidemic natural measles Parenteral inoculation of attenuated virus was followed by reactions consisting of fever, rash or both. The fever lasted from one to three days and occasionally reached 105°F. Rash was of the limited type usually associated with cases of measles which have been modified by the administration of gamma globulin It occurred in roughly half of the vaccinees. Koplik's spots have been detected in about 20% of children receiving the attenuated strain. The recipients rarely required bed rest and frequently remained at play in a normal fashion. In certain children the entire "reaction" might have been missed without prior warning Neutralizing and complement-fixing antibodies developed in all persons showing clinical signs and have also been reported in some who remained asymptomatic. Virus could not be recovered from the blood or throat washings of vaccinated individuals nor was there clinical or serological evidence of infection in susceptible contacts These studies are continuing and promise contributions not only of significance to the pathogenesis of measles but perhaps to our understanding of its rapid epidemic spread as well.

C. Pathologic Changes Induced by Measles Virus; Giant Cell Pneumonia

The cytologic alterations accompanying experimental measles infection of cell cultures are variable and may be dependent upon virus strain, passage history, a specific cell type, or even the medium used for cell nutrition (Black *et al.*, 1959). On the other hand, in the intact animal the appearance of multinucleate giant cells constitutes the hallmark of tissue involvement, particularly of the lymphoid system. These cellular aggregates do not persist throughout the disease but probably appear prior to the appearance of a rash and may disappear shortly after (Roberts and Bain, 1958).

In epithelial cell monolayers of man, monkeys, or rodents, virulent measles virus induces the appearance of two types of change The first consists of large focal aggregates formed by the fusing of cytoplasm into a giant cell or "syncytia" (Bech and Von Magnus, 1958) Eosinophilic bodies are readily visible in both the cytoplasm and nucleus As the culture ages, vacuolization of the syncytium progresses and the entire sheet may become destroyed

The second, or fibroblastic, type of cytopathic effect has been described in human amnion cells (Milanovic *et al*, 1957) and in serial passage in the HEP-2 line of human malignant epithelium (Reissig *et al*, 1956). Referred to as "spindle cell transformation" by the Enders group, it consists of a diffuse alteration of the rounded epithelial components of the monolayer into an elongated, fusiform, stellate cell, often with multiple pseudopodial strands at the poles. Both giant and spindle cells can develop in the same tissue culture and this is not an uncommon finding Seligman and Rapp (1959) have suggestive evidence that giant cell formation is a property of genetically differentiated particles in the strain and is associated with the natural, "wild type" of measles virus. Using electron microscopy, Reissig (1958) observed that the measles inclusion body is an area of low electron density devoid of the normal chromatin material She concluded that it does not represent an aggregation of virus material but rather a degenerative type of lesion resulting from infection

The etiology of giant cell pneumonia, an interstitial pneumonitis first described in children by Hecht (1910), has long been a source of speculation. Because the cytologic alterations seen in giant cell pneumonia are also found in measles in man and canine distemper infection in dogs, these viruses have been proposed as the etiologic agents of Hecht's disease The situation has recently been considerably clarified first, by the successful isolation of measles virus from three fatal cases

of giant cell pneumonia in children in whom clinical measles was not observed (*Enders et al*, *1959b*) and second, *by the isolation of measles* from two fatal and two nonfatal cases of pneumonitis secondary to measles (Mitus *et al.*, 1959). The strains obtained from these patients were identical with the Edmonston virus in growth characteristics, host range, and serologic behavior. An interesting and unusual feature of the two fatal cases was the persistence of virus in the oropharynx and the patients' failure to develop antibody. Whether other agents, such as canine distemper virus, can also cause the Hecht syndrome remains to be determined.

D. The Serologic Pattern of Measles Infection in Man

The patterns of complement-fixing and neutralizing antibodies which develop after measles in children and adults have been described by Enders *et al.* (1957), Ruckle and Rogers (1957), and Girardi *et al.* (1958)

Recent work has been directed at the clarification of measles epidemiology with the aid of the *in vitro* serological tool provided by tissue culture. The degree of correlation between reported cases of measles and the development of antibody in an urban population (New Haven, Connecticut) was investigated by Black (1959b). As anticipated, children with a history of measles nearly always had positive serology, 98%; however, a significant proportion, 12 of 37, of persons who claimed they had never experienced the disease had both positive complement-fixing and neutralization tests although with a lower mean value than in the group of overt cases The relative susceptibility of the 1- to 4-year-old age group is well illustrated in Fig 1 taken from Black's paper.

It has generally been accepted that the prophylactic administration of gamma globulin to measles susceptible individuals confers only a transient immunity unless some evidence of infection subsequently becomes manifest in the recipient. Black and Yannet (1960) have studied the antibody patterns and measles epidemiology in susceptible, institutionalized children who were given gamma globulin shortly after exposure to a case of measles. Neutralizing and CF antibodies appeared in the sera of 19 of 38 individuals following exposure, although none of these had clinical signs of infection. When 5 of these 19 children were re-exposed, 7 months later, all were immune in spite of the fact that antibody levels resulting from globulin-suppressed measles were lower than those resulting from overt disease. Black suggests that administration of gamma globulin not only reduces the severity of subsequent measles but also *diminishes the extent of its contact spread.*

In unpublished studies carried out in collaboration with Dr. Leon

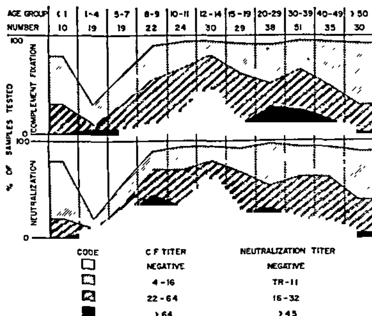


Fig 1 Measles antibodies by age and titer (from Black, 1959b)

Rosen, we have noted a similar appearance of complement-fixing antibody in the sera of 11 healthy inmates of a children's home who were residents at the time of a measles outbreak in the community but who remained asymptomatic during this period Each of the children was under close daily medical scrutiny, and no clinical signs whatever were observed between the time of collection of the pre-, or baseline, and postepidemic serum specimens In the sera of these children taken approximately 7 months after the end of the epidemic, complement-fixing antibody levels ranged from 1 . 30 to 1 256

The high contagiousness of measles is well exemplified by Black's (1959b) finding that only 10% or less of adults were devoid of antibody to this virus. In preschool groups, the rate of appearance of antibody was greatly influenced by the size of the family, an observation in accord with the experience of most pediatricians In an epidemic of measles in Greenland described by Bech (1959), 1203 persons in a small community had the disease during a 6-week period and only 4 theoretically susceptible persons remained free of symptoms Bech noted that a rise of CF titer followed closely after the appearance of rash, a finding somewhat at variance with that of most other observers who describe a 3- to

5-day interval between the onset of clinical signs and increase in measles antibody. A few of the sera were tested against both the "Edmonston" virus and strains isolated in Denmark, but no evidence of differences between measles strains was obtained. When sera from immune adult relatives exposed to sick children were tested, no evidence of a booster phenomenon was observed during the time of contact and 10 to 16 days later, suggesting that the prolonged elevation of antibody in man may not necessarily be the resultant of repeated "recall" exposures.

III. Evidence for the Relationship of Measles and Canine Distemper Viruses

Increasing attention is being given to the possibility of human infections with canine distemper virus, a consideration first proposed by Bryan (1928) and Nicolle (1931) and subsequently by Pinkerton *et al.* (1945) Within the past 6 years, the problem has been approached experimentally and this has been facilitated by the adaptation of canine distemper, first to embryonated eggs by Haig (1948), Cabasso and Cox (1949), and later to suckling mice by Morse *et al* (1953) and Carlström (1957). In addition, the growth of measles virus in several types of tissue culture now provides the worker with a complete set of diagnostic tools for investigating this area beyond the earlier observations of histopathologic and clinical similarities

Studies on this problem fall into three major categories:

1. The description of clinical and pathologic similarities between natural and experimental infections with canine distemper and measles.
2. The establishment of the canine distemper and measles antibody patterns in man as correlated with past or recent measles infection
3 Immunologic investigation of experimental measles and/or canine distemper in several experimental hosts.

A. Similarities in Pathologic Effects

Adams and his associates (1953, 1956) play a prominent role in this field and have emphasized similarities in the inclusion bodies and giant cells found in canine distemper and measles-infected tissues. Along these same lines, Rockborn (1958) described syncytium formation in dog kidney tissue cultures infected with canine distemper virus identical to that described previously by Enders *et al.* (see above). The thesis originally advanced by Adams and his collaborators that canine distemper may be a specific cause of an acute respiratory illness in children must now be weighed against the actual isolation of measles virus from acute pneumonitis in children. The demonstration of canine distemper virus in human clinical material is an accomplishment which has not yet

been reported. In this connection the proposed utilization of fluorescein-labeled distemper specific antibodies to demonstrate antigen in tissues appears to be a promising approach (Adams, 1959) In conclusion, it may be well to stress the observation of Plowright and Ferris (1959) that the formation of giant cell syncytia is not only associated with measles and canine distemper but is a characteristic shared by a large number of viruses including mumps, several latent agents isolated from monkey kidney, parainfluenza strains, rinderpest, and lumpy-skin disease of cattle.

B. Antibody Patterns in Man

Most human sera acquire the capacity to neutralize canine distemper virus (CDV) early in adolescence This was demonstrated by Karzon (1955), who studied the ability of sera from 266 individuals to inactivate relatively small amounts, 50 EID_{50}, of canine distemper virus Antibody titers in positive individuals ranged between 1 5 and 1 640 with a mean of 1:40. Human gamma globulin was equal to canine convalescent hyperimmune serum in its neutralizing antibody level in canine distemper Similar findings have been reported by Imagawa *et al* (1954) While evidence that this represented true antibody was fairly convincing, Karzon could find no correlation between titer and a history of any specific infection, including measles This observation is not entirely consonant with the more recent studies of Carlstrom in Sweden, who has employed suckling mouse-adapted strains of canine distemper and measles viruses for neutralization studies in order to ensure the same host system (1957, 1959a, 1959b) In her determinations of cross-neutralization, Carlstrom used an amount of virus just sufficient to cause 100% mortality End points were considered as the highest dilution (5-fold series) protecting 50% of the test animals On this basis significant rises in antibody levels against canine distemper were found with the paired acute and convalescent sera from 16 measles patients while no correlation was found between a past history of several other viral diseases and the capacity to inactivate distemper When the antibody titers for measles and canine distemper were compared in the same serum, whether from measles-convalescent or random selected children, that to measles was always present in the greater amount in a ratio of about 1·25.

In the course of investigating the pathogenicity of attenuated strains of CDV for man, we have recently screened the sera of children who had no prior history of measles for their level of canine distemper neutralizing antibody (Millian *et al*, 1960) Using the sera of 36 persons between 2 and 12 years of age, the test was performed in chick embryos with 200

5-day interval between the onset of clinical signs and increase in measles antibody. A few of the sera were tested against both the "Edmonston" virus and strains isolated in Denmark, but no evidence of differences between measles strains was obtained When sera from immune adult relatives exposed to sick children were tested, no evidence of a booster phenomenon was observed during the time of contact and 10 to 16 days later, suggesting that the prolonged elevation of antibody in man may not necessarily be the resultant of repeated "recall" exposures.

III. Evidence for the Relationship of Measles and Canine Distemper Viruses

Increasing attention is being given to the possibility of human infections with canine distemper virus, a consideration first proposed by Bryan (1928) and Nicolle (1931) and subsequently by Pinkerton *et al.* (1945). Within the past 6 years, the problem has been approached experimentally and this has been facilitated by the adaptation of canine distemper, first to embryonated eggs by Haig (1948), Cabasso and Cox (1949), and later to suckling mice by Morse *et al* (1953) and Carlström (1957). In addition, the growth of measles virus in several types of tissue culture now provides the worker with a complete set of diagnostic tools for investigating this area beyond the earlier observations of histopathologic and clinical similarities

Studies on this problem fall into three major categories:

1. The description of clinical and pathologic similarities between natural and experimental infections with canine distemper and measles.
2 The establishment of the canine distemper and measles antibody patterns in man as correlated with past or recent measles infection
3 Immunologic investigation of experimental measles and/or canine distemper in several experimental hosts.

A. Similarities in Pathologic Effects

Adams and his associates (1953, 1956) play a prominent role in this field and have emphasized similarities in the inclusion bodies and giant cells found in canine distemper and measles-infected tissues. Along these same lines, Rockborn (1958) described syncytium formation in dog kidney tissue cultures infected with canine distemper virus identical to that described previously by Enders *et al* (see above). The thesis originally advanced by Adams and his collaborators that canine distemper may be a specific cause of an acute respiratory illness in children must now be weighed against the actual isolation of measles virus from acute pneumonitis in children The demonstration of canine distemper virus in human clinical material is an accomplishment which has not yet

TABLE II

Canine Distemper and Measles Antibody in Children Following Administration of Either Live or Formaldehyde-Inactivated Canine Distemper or Measles Virus

Serum number	Vaccine administered	Pre-vaccination	Canine Distemper Neutralizing 3 wk	4 wk	8 wk	12 wk	Measles CF 3 wk
41*	Formaldehyde-inactivated canine distemper vaccine	22	—	142	20	10	
20*		39	—	132	22	10	
187*		12	—	125	21	10	
22*		18	—	111	10	10	<16
15*		26	—	61	12	10	
	Geometric mean titer	22	—	110	16	10	
178*	Live canine distemper virus, egg-adapted	10	—	282	56	10	
195*		61	—	282	15	33	
159*		10	—	215	21	16	
230*		NA	—	175	38	10	
77*		10	—	145	18	29	
57*		NA	—	135	60	18	
161*		32	—	132	100	10	
197*		NA	—	130	26	10	
188*		40	—	125	100	NA	
190*		10	—	125	68	10	
158*		16	—	100	124	10	
186*		36	—	100	12	31	<16
181*		14	—	19	110	10	
60		NA	332	—	—	—	
59		23	282	—	—	—	
58		13	246	—	—	—	
68		NA	163	—	—	—	
67		30	132	—	—	—	
69		36	100	—	—	—	
55		NA	71	—	—	—	
64		10	56	—	—	—	
56		33	51	—	—	—	
66		NA	45	—	—	—	
61		21	23	—	—	—	
65		NA	16	—	—	—	
62		10	10	—	—	—	
	Geometric mean titer	20	73	129	45	15	
48	Live measles virus (attenuated)	35	502	—	—	—	32
42		45	276	—	—	—	NA
44		10	100	—	—	—	32
41		13	71	—	—	—	<16
50		26	38	—	—	—	<16
	Geometric mean titer	22	130	—	—	—	

NA = None available

* = Received second and third doses immediately after 4- and 8-week bleedings

to 300 EID_{50} of canine distemper virus, a larger challenge dose than that employed by either Karzon or Carlström. The prevaccination sera of these children had distemper antibody titers ranging from 1 : 10 to 1 : 60. Each of these individuals was then inoculated with either formaldehyde-killed or live, egg-adapted, attenuated canine distemper virus, as shown in Table II Three to four weeks following the subcutaneous injection of either living or killed virus there was an increase in titer in the majority of sera ranging up to 1 : 332; the postvaccination geometric mean was 1 : 100 Five children who received killed distemper and 13 of those vaccinated with living virus were also given a second and third inoculation of the same material, but there was no further increase in antibody, *rather a decline to prevaccination levels continued* *As can* be seen, the measles complement-fixing titer of all the children was less than 1 : 16 in prevaccination specimens and was not increased beyond this level after a moderate antigenic stimulation by canine distemper virus.

Sera were also available from a group of 5 children who had been subcutaneously inoculated three weeks previously with the attenuated Edmonston strain of measles virus and in whom mild clinical measles had developed. Three of these five children acquired a level of canine distemper antibody comparable to that elicited in other children by canine distemper vaccine (see Table II). Measles antibody titers increased to 1 : 32 in 2 of the 4 available sera, and both of these were from patients who had developed appreciable canine distemper titers

The antigenic stimulus for canine distemper virus antibody in man remains unknown Each investigator has used a different strain of canine distemper and different procedures for antibody investigation While the association of distemper antibody development with measles infection appears established, the significance of the observation should become clearer as more information becomes available on serologic patterns obtained with specific hyperimmune animal sera using more quantitative and sensitive techniques

C. Immunologic Studies in Animals

We have attempted to combine and summarize the available information on antigenic overlap between canine distemper and measles viruses in Table III. Although each study involves technical details which may well have a bearing upon contradictory observations, it is not possible to include tabulation of these variables in a condensation of this type. Both resistant (rabbit, chicken) and susceptible (dog, ferret) species have been used as sources of canine distemper virus antibody; a similar situation holds for measles We consider this factor to

be of significance since it is quite conceivable that the susceptible host will respond with a serum of broader specificity than the nonsusceptible animal which is essentially being artificially immunized In an equally diverse manner, antigens have been produced in rodents, eggs, or tissue cultures, and in varying titers The tests have involved differences in incubation time, temperature, and in the designation of an end point Nevertheless, careful reading of the original reports reveals several points of basic and probably significant agreement Homologous neutralization is considerably in excess of the heterologous titer or, when equal levels are reported, they are relatively low (less than 1:10), with the exception of one hyperimmune dog serum tested by Carlström (1959a) When a canine distemper immune serum cross-neutralizes measles virus, it only does so to a degree considerably below its homologous titer Exposure of the ferret and dog to measles virus modifies their response to a canine distemper virus challenge but there is not complete cross-immunity.

Recently completed studies on the behavior of canine distemper in monkeys, by Schwarz *et al*, not only provide the first extensive work on the multiplication of this agent in primates, but also illustrates the inability of high levels of distemper or measles antibody to interfere with multiplication of the heterologous virus.* In Table IV is shown the findings on 6 cynomolgus monkeys which received distemper vaccine and responded with antibody titers between 1:200 and 1 1000 They were then inoculated with measles virus by the intranasal and intramuscular route. Neither an accelerated nor elevated pattern of measles antibody resulted from prior distemper exposure. However, as in the case of distemper-vaccinated dogs (Warren *et al*, 1960), measles antigen appeared to stimulate distemper titers so that end points by the third week ranged between 1:10,000 and 1:1,000,000 While these high levels compare favorably with those that follow a booster dose of distemper in distemper immune monkeys, it should be emphasized that no measles crossing was observed in animals hyperimmunized with the canine virus alone

Data from a converse experiment in which measles immune monkeys were challenged with distemper virus is seen in Table V. The curve of distemper antibody development was not unlike that occurring in "virgin," untreated monkeys These experiments gain in importance not only because they were performed in susceptible primates but also because live virus was used and multiplication occurred in the presence of high titers of the heterologous antibody

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TABLE III

EXPERIMENTAL DATA RELATIVE TO THE ANTIGENIC CROSSING OF MEASLES AND CANINE DISTEMPER VIRUSES

Assay	Source CDV		Source Measles		Observation	Reference
	Virus	Antiserum	Virus	Antiserum		
	Mouse-adapted	Ferret	HeLa TC	—	Sera neutralized measles CPE to 1 8	Adams *et al*, 1957
	Egg-adapted	Ferret				
	Mouse-adapted	—	HeLa TC	Ferret	Sera neutralized CDV deaths 1.4	
	Mouse-adapted	Rabbit	Mouse-adapted	—	Sera neutralized measles and CDV to same, but low titers	Carlstrom, 1959a
	Mouse-adapted	—	Mouse-adapted	Rabbit	Sera neutralized measles (homologous) to 5× greater titer than CDV	
	Ferret-adapted	Dog	Mouse-adapted	—	Serum (1) neutralized both to 1.125	
	Egg-adapted	Dog	HeLa TC	—	None of 10 vaccinated or convalescent puppies neutralized measles virus	Cabasso *et al*, 1959b
Neutralization and/or complement fixation	Egg-adapted	Chicken	HeLa TC	Chicken	No neutralization obtained with the heterologous agent	
	Egg-adapted	—	Monkey kidney TC	Dog	Some neutralization of CDV after measles convalescence but accelerated CDV rise after CDV challenge	Warren *et al*, 1960
	Egg-adapted	Dog	Monkey kidney TC	—	None of 8 vaccinated puppies fixed complement with measles	
	Egg-adapted	—	HeLa TC	Monkey	No neutralization of CDV in presence of high measles antibody in 2 monkeys	Cabasso, *et al*, 1959b
Protection	Ferret	—	HeLa TC	—	Partial protection against CDV conferred by live measles vaccination of ferrets	Adams and Imagawa, 1957
	Ferret	—	Monkey kidney or human heart ——	—	Partial protection against CDV conferred by live measles vaccination of dogs	Warren *et al*, 1960

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TABLE V

ANTIBODY FOLLOWING INTRAMUSCULAR INOCULATION OF LIVE DISTEMPER VACCINE INTO CYNOMOLGUS MONKEYS PREVIOUSLY INOCULATED WITH MEASLES VACCINE*

Monkey No	Antibody titers											
	Day of inoculation			1-Wk postinoculation			2-Wk postinoculation			3-Wk postinoculation		
	Measles		Distemper	Measles		Distemper	Measles		Distemper	Measles		Distemper
	CF	Neut	Neut	CF	Neut	Neut	CF	Neut	Neut	CF	Neut	Neut
247	1 512	1 32	1 10	1 256	1 320	1 500	1 256	1 320	1 32,000	1 128[a]	1 320[a]	1 32,000
248	1 256	1 320	1 100	1 512	1 320	1 500	1 512	1 320	1 1600	1 256	1 320	1 10,000
257	1 1024[b]	1 200	1 50	1 512	1 320	1 50	1 512	1 320	1 500	1 256	1 320	1 1000

* *Data from* Schwarz *et al* To be published

[a] Titers of 4-wk postinoculation sera

[b] *Titer of serum* 7 days prior to inoculation

Distemper vaccine titer ≃ $10^{5.5}$ EID_{50}

TABLE IV

Antibody Patterns Following Inoculation of Live Measles Vaccine into Cynomolgus Monkeys Previously Inoculated with Distemper Vaccine[a]

Monkey No	Inoculation		At inoculation		1-Wk postinoculation		2-Wk postinoculation		3-Wk. postinoculation	
	Route	Amount	Measles	Distemper	Measles	Distemper	Measles	Distemper	Measles	Distemper
251	I M	$10^{3.2}$	0	1:500	0	1:10,000	1:32	1:100,000	1:320	1:100,000
255	I M	$10^{5.2}$	0	1:1000	0	1:400,000	1:10	10^6	1:32	1:10^6
73	I M	$10^{3.2}$	0	1:200	0	1:10^6	1:32	1:10^6	1:320	1:10^6
229	I M	$10^{3.2}$	0	N T[b]	0	N T	1:2	N T	1:20	N T
253	I N and oral	$10^{5.2}$	0	1:320	0	1:50	1:32	1:10,000	1:32	1:10,000
254	I N and oral	$10^{5.2}$	0	1:320	0	1:100	1:32	1:10,000	1:320	1:10,000

[a] Data from Schwarz *et al.*, (1960) To be published
[b] N T. = Not tested.

cell cultures (Lasfargues and Corvazier, 1952; Cabasso *et al*, 1959a; Karzon and Bussel, 1959) provide materials of the same order of infectivity as the parent canine or avianized virus Using culture fluids taken from twentieth to fortieth culture passages, Cabasso *et al.* (1959a) have prepared attenuated vaccines capable of protecting ferrets, thus demonstrating retention of immunogenicity with decrease in virulence.

Recent findings suggest a relation between the cytopathic effect of distemper virus and its source and host cell The strain of distemper, adapted by Rockborn (1958) to dog kidney tissue culture, was obtained from the tissues of a naturally infected dog It produced a vacuolar degeneration in canine cell cultures accompanied by the appearance of granular intracytoplasmic inclusions Syncytium formation similar to that of measles was commonly observed In contrast to virus grown on avian cells, after 24 passages the canine kidney-adapted line of distemper remained undiminished in its virulence for dogs

Both of the classic egg-adapted strains of canine distemper have also been propagated in avian cells but with strikingly different cytopathology Using the Lederle strain, Cabasso *et al* (1959a) were able to passage the virus in an extended series, but its presence could only be demonstrated by subinoculation into embryonated eggs In contrast, the Onderstepoort strain not only caused rapid destruction of chick embryo monolayers but the CPE consisted of rounding and necrosis of the cells; the occurrence of syncytia or giant cell formation is not reported (Karzon and Bussel, 1959) The latter author observed that the Lederle strain also could induce cytopathic changes if it were grown in a medium containing bovine amniotic fluid and bovine serum but not in medium 199 with horse serum However, the Onderstepoort virus was cytopathogenic regardless of the culture medium

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this group of agents appear to be related to virus strain, cell substrate, and cultural conditions

B Immunologic Response of Dogs to Distemper Infection

The problems associated with the occasional failure of potent canine distemper vaccine to protect exposed dogs have been investigated by Gillespie *et al* (1958) and Rockborn (1958) Using the *in ovo* procedure, the curve of development of neutralizing antibody *following* inoculation of virulent distemper virus has been found to rise fairly rapidly and remain elevated for at least 2 years [see Fig 2 (from Gillespie *et al*, 1958)] A similar pattern is found in the naturally con-

On the whole, the available evidence is in agreement with the conclusion derived from the analysis of human antibody patterns, i.e., we are dealing with strains of virus sharing only some of their antigenic components. This is reflected to a varying degree depending upon the test system and species and may be comparable to the situation existing between strains of influenza viruses.

IV Canine Distemper Virus

A comprehensive description of canine distemper can be found in W. A Hagan and D. W. Bruner's "The Infectious Diseases of Domestic Animals," 3rd ed, Cornell University Press, 1957. For an exhaustive, if noncritical, review see L. F. Whitney and G D. Whitney's "The Distemper Complex," Practical Science Publishing Co., Orange, Connecticut, 1953 A good concise summary of canine distemper infection in tissue cultures can be found in Rockborn (1958).

A. Cultivation

Considering the importance of canine distemper in veterinary medicine, one is struck by the relatively few investigations that have contributed to the characterization of this virus. To some extent, this may have been due to the failure of canine distemper to adapt readily to cells in culture. Thus, serological or biophysical studies have been restricted by time-consuming titrations in eggs, dogs, or the ferret. Within the past few years, this situation has been improved by the ready adaptation of canine distemper virus to suckling mice (Morse, 1953, Carlström, 1956) and suckling hamsters (Cabasso and Cox, 1955), although the ferret still appears to be the conventional test host for virus isolation and propagation of highly virulent material (Larin, 1955). From the descriptions of the course of canine distemper infection in these suckling rodents, it would be difficult to distinguish it clinically from measles infection in the same species (Carlström, 1959a; Burnstein *et al*, 1958). Virus titers vary between 10^{-2} to 10^{-3} and are comparable to those obtained in embryonated eggs. Because suckling mice are a readily available species developing overt disease with both measles and canine distemper, they have, as described above, been employed by Carlstrom for crossneutralization studies with specific antisera.

Cultivation of canine distemper virus in tissue culture has been difficult because under the conditions employed the agent produced no or minimal cytopathic effect, although multiplication could be demonstrated in serial passages by inoculation of culture fluid into ferrets or embryonated eggs. *Renal, testicular, and pulmonary epithelium from* dogs (Dedie and Klopötke, 1952; Rockborn, 1958) and chick embryo

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Recent findings suggest a relation between the cytopathic effect of distemper virus and its source and host cell The strain of distemper, adapted by Rockborn (1958) to dog kidney tissue culture, was obtained from the tissues of a naturally infected dog It produced a vacuolar degeneration in canine cell cultures accompanied by the appearance of granular intracytoplasmic inclusions Syncytium formation similar to that of measles was commonly observed In contrast to virus grown on avian cells, after 24 passages the canine kidney-adapted line of distemper remained undiminished in its virulence for dogs

Both of the classic egg-adapted strains of canine distemper have also been propagated in avian cells but with strikingly different cytopathology. Using the Lederle strain, Cabasso *et al* (1959a) were able to passage the virus in an extended series, but its presence could only be demonstrated by subinoculation into embryonated eggs In contrast, the Onderstepoort strain not only caused rapid destruction of chick embryo monolayers but the CPE consisted of rounding and necrosis of the cells, the occurrence of syncytia or giant cell formation is not reported (Karzon and Bussel, 1959) The latter author observed that the Lederle strain also could induce cytopathic changes if it were grown in a medium containing bovine amniotic fluid and bovine serum but not in medium 199 with horse serum However, the Onderstepoort virus was cytopathogenic regardless of the culture medium

In the light of these recent observations and the older report of Reissig *et al* (1956), describing the relation of glutamine concentration to giant cell formation by measles virus, the cytopathic alterations caused by this group of agents appear to be related to virus strain, cell substrate, and cultural conditions

B *Immunologic Response of Dogs to Distemper Infection*

The problems associated with the occasional failure of potent canine distemper vaccine to protect exposed dogs have been investigated by Gillespie *et al* (1958) and Rockborn (1958). Using the *in ovo* procedure, the curve of development of neutralizing antibody following inoculation of virulent distemper virus has been found to rise fairly rapidly and remain elevated for at least 2 years [see Fig 2 (from Gillespie *et al.*, 1958)] A similar pattern is found in the naturally con-

On the whole, the available evidence is in agreement with the conclusion derived from the analysis of human antibody patterns, i e., we are dealing with strains of virus sharing only some of their antigenic components. This is reflected to a varying degree depending upon the test system and species and may be comparable to the situation existing between *strains of influenza viruses.*

IV Canine Distemper Virus

A comprehensive description of canine distemper can be found in W. A Hagan and D. W. Bruner's "The Infectious Diseases of Domestic Animals," 3rd ed, Cornell University Press, 1957. For an exhaustive, if noncritical, review see L F. Whitney and G. D Whitney's "The Distemper Complex," Practical Science Publishing Co., Orange, Connecticut, 1953 A good concise summary of canine distemper infection in tissue cultures can be found in Rockborn (1958).

A. Cultivation

Considering the importance of canine distemper in veterinary medicine, one is struck by the relatively few investigations that have contributed to the characterization of this virus. To some extent, this may have been due to the failure of canine distemper to adapt readily to cells in culture. Thus, serological or biophysical studies have been restricted by time-consuming titrations in eggs, dogs, or the ferret. Within the past few years, this situation has been improved by the ready adaptation of canine distemper virus to suckling mice (Morse, 1953; Carlström, 1956) and suckling hamsters (Cabasso and Cox, 1955), although the ferret still appears to be the conventional test host for virus isolation and propagation of highly virulent material (Larin, 1955). From the descriptions of the course of canine distemper infection in these suckling rodents, it would be difficult to distinguish it clinically from measles infection in the same species (Carlström, 1959a; Burnstein *et al*, 1958). Virus titers vary between 10^{-2} to 10^{-3} and are comparable to those obtained in embryonated eggs. Because suckling mice are a readily available species developing overt disease with both measles and canine distemper, they have, as described above, been employed by Carlström for cross-neutralization studies with specific antisera.

Cultivation of canine distemper virus in tissue culture has been difficult because under the conditions employed the agent produced no or minimal cytopathic effect, although multiplication could be demonstrated in serial passages by inoculation of culture fluid into ferrets or embryonated eggs. Renal, testicular, and pulmonary epithelium from dogs (Dedie and Klopötke, 1952; Rockborn, 1958) and chick embryo

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tracted disease. One interesting exception to this is the discovery by Rockborn of a canine distemper strain (Thun) antigenically identical to classic distemper virus but to which antibody responses were considerably delayed in both the dog and ferret.

Transplacental and colostral transfer of distemper antibody occurs and not only protects puppies from exposure during the first few weeks of life, but may also nullify the effectiveness of vaccination with live virus during this period. A high level of distemper antibody is widely prevalent in adult dog populations. This seems reasonably conclusive evidence that subclinical infection occurs with canine distemper; in this respect, it resembles the epidemiologic pattern of measles in man.

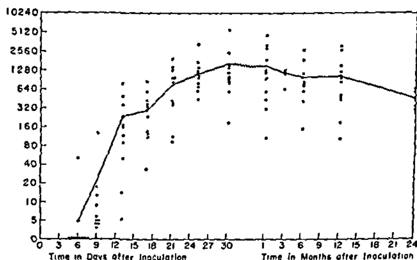


FIG 2 The development and persistence of neutralizing antibodies in dogs inoculated with virulent distemper virus (from Gillespie *et al*, 1958)

The nature of the prolonged viremia present in most distemper-infected animals has been studied by Rockborn (1958), who presents data suggesting that it is dependent upon the intracellular localization of virus in cells of the reticuloendothelial system which protect the agent against circulating neutralizing antibody—a finding in agreement with the earlier observations of Liu and Coffin (1953) that canine distemper virus localizes in the lymphatic system

C. Strain Variation

Variation in the severity and course of infection caused by antigenically identical distemper isolates has been a troublesome problem

since 1926 when Laidlaw and Dunkin first utilized the ferret as the host of choice for work with this virus (see Whitney and Whitney (1953) for a review of the extensive literature of this period). Recent reports have begun to characterize these variations in greater detail. The isolation by Rockborn (1958) of one strain slow to stimulate antibody in dogs has already been cited. Serial egg passage rapidly alters the pathogenicity of natural strains to the point where they may be used as attenuated vaccines, as reported a decade ago by Haig (1948), and by Cabasso and Cox (1949) A very extensive survey was made by Larin (1955), who studied the behavior of 55 different canine distemper isolates taken from moribund or dead dogs in Great Britain Virulence for ferrets varied among strains which were fatal within 10 to 14 days, strains which caused a prolonged illness with varying fatality, and those inducing a prolonged relapsing fever with few deaths Since certain of these strains produced no cross-immunity in ferrets to other lines of distemper, and in the absence of serologic observations, we must be cautious in accepting Larin's conclusions that "canine distemper is not caused by a homogenous virus, but by several types which are not stable entities with predictable behavior"

In a collaborative study, we have encountered suggestive evidence that different strains of canine distemper virus may vary in their degree of cross-reactivity with measles (Warren *et al*, 1960) When dogs were immunized with canine distemper of canine, ferret, or egg origin, the level of distemper and measles antibody obtained was found to vary and possibly relate to the strains and the host tissue from which they were propagated, as shown in Table VI In each instance, the infective titer of the strains used was about the same These are only preliminary findings, but if strain variations in antigenicity are found to exist, such variations will have to be taken into account in the performance of serologic tests with the viruses of the measles group

D The Use of Canine Distemper Virus for Immunization against Measles

As a consequence of the finding that neutralizing antibodies for distemper are ubiquitous in certain adult human populations and on the supposition that such antibody might confer protection against measles virus, several workers have attempted to immunize children and adults with live canine distemper vaccine using egg-adapted attenuated virus Adams *et al.* (1959) have briefly reported on an experiment begun in 1952 in which 200 individuals of unstated age in a state mental hospital were injected with a single 1-ml dose of live, egg-adapted (Lederle) distemper vaccine The clinical measles attack rate over the next 5

TABLE VI

ANTIBODY TO CANINE DISTEMPER (NEUTRALIZING) AND MEASLES VIRUS (CF) FOLLOWING INFECTION OF YOUNG LITTER MATE DOGS WITH VARIOUS STRAINS OF CANINE DISTEMPER*

Strain	Source	Titer/ml	Dog No	Preinfection		Postinfection Antibody To.			
						Canine distemper		Measles	
				CD	Measles	10 Days	20 Days	10 Days	20 Days
Wisconsin	Raccoon spleen	(Log) 5.3[b]	201	<1 10	<1.16	1.240	1.800	1.16	1·32
			211	<1 10	<1.16	1.240	>1 800	1.16	1:16
Onderstepoort	Egg CAM	4 0[b]	202	<1 10	<1 16	1 60	1 100	<1 16	1.16
			212	<1.10	<1 16	<1 40	1 200	<1.16	<1.16
Onderstepoort	Ferret spleen	6.3[c]	203	<1 10	<1.16	1.40	1·600	1:16	1 64
			213	<1 10	<1.16	1 240	>1.800	1.16	1:32
Lederle	Egg CAM	5 0[b]	204	<1 10	<1 16	<1·40	<1:100	<1.16	<1 16
			214	<1.10	<1 16	1.40	<1:100	<1:16	<1·16
Lederle	Ferret spleen	5 0[c]	205	<1 10	<1:16	1:60	<1:100	<1:16	1·16
			215	<1:10	<1.16	ND	<1.100	<1:16	<1.16
RL	Egg CAM	5 0[b]	206	<1·10	<1:16	1:80	1·150	1:128	>1:128
			216	<1:10	<1.16	<1.40	1:600	<1:16	<1:16

* Infecting dose, 1 0 ml. of 10% tissue suspension subcutaneous
[b] Virus titer determined in chick embryo
[c] Virus titer determined in ferret

years was 1.8% in 165 vaccinated subjects and 5 9% in 1190 nonvaccinated controls No antibody determinations are reported and most of the measles cases developed during an epidemic which was prevalent 3 years from the time vaccination was introduced. Based on the observations of Millian *et al.* (1960), a single dose of canine distemper vaccine would not be expected to induce appreciable canine distemper antibody and the low titers which do appear would not be likely to persist after such a long interval.

Through the kindness of Drs. A J. F. Schwarz and M. Hoekenga, we have been supplied with a prepublication summary of an investigation of the effectiveness of distemper vaccination on the course of a measles outbreak in Panama In that country, migrant labor recruited from rural regions, particularly among isolated Indian settlements, constitutes a highly susceptible population in whom measles epidemics are cyclic. In March, 1959, a lyophilized distemper vaccine was administered to 388 persons, using a dose of 0 5 ml for children and 1 0 ml for persons over 5 years of age. There were no reactions to the live vaccine. Table VII illustrates the measles attack rate, 14 cases (3 6%) in the vaccinated group and 25 (6 0%) in the controls The authors conclude that this difference in attack rate does not suggest that a significant degree of protection for man is conferred by a single dose of distemper vaccine

TABLE VII
OCCURRENCE OF MEASLES IN INDIVIDUALS VACCINATED WITH TISSUE CULTURE DISTEMPER VACCINE*

	Vaccinated			Control		
		Measles			Measles	
Age group	No of persons	Cases	%	No of persons	Cases	%
Under 2 yr	80	5	4 1	53	5	12 1
2– 5 yr	141	4	4 1	63	9	12 1
6–10 yr	80	3	3 1	58	3	4.0
11–15 yr	18	0	3 1	41	1	4 0
16–20 yr	30	0	3 0	61	3	3.5
21–25 yr	21	0	3 0	73	3	3.5
26–30 yr	18	1	3 0	65	1	3.5
Totals	388	14	3 4	414	25	6 0

Period of observation was 3–4 months

* Data from Hoekenga and Schwarz To be published

V. Rinderpest Virus

A. Cultivation in Tissue Culture

In addition to ruminants, its natural hosts, rinderpest virus will multiply in embryonated eggs, rabbits, mice, and hamsters. The successful cultivation of the virus in trypsinized monolayers of bovine kidney by Plowright and Ferris (1957, 1959) has not only made available attenuated strains of virus but has also provided a means for further delineation of the cytopathic effects of this relatively little studied agent and facilitated work on its cross-relationships. Multiplication in culture was rapid; titers of 10^3 to 10^4 TCD_{50}/ml being often reached by the second to fourth days after infection. Virus continued to be released for several weeks in a manner reminiscent of the growth of measles in monkey or human cell cultures. Calf testis, sheep kidney and testis, goat, pig, and hamster kidney cultures were all destroyed by rinderpest virus after its adaptation to bovine cell culture. However, two attenuated strains, one from goats and one from rabbits, could not be successfully cultivated in bovine cell cultures, suggesting that a basic, perhaps genetically selective alteration in tissue tropism may be associated with attenuation via caprine or rodent passage.

Degenerative changes in tissue cultures included the formation of stellate cells with increased refractility and the appearance of giant cells or syncytia and vacuolization indistinguishable from those of measles. Small eosinophilic inclusions were plentiful in older cultures and were surrounded by an opaque halo, reminiscent of the description by Rockborn (1958) of similar inclusions in his dog kidney cultures of distemper virus.

B. Serologic Reactions between Rinderpest and Measles in Vitro

Human antibody patterns to distemper show a high, but not perfect, correlation with a history of measles infection as discussed in earlier sections of this review. Comparable neutralization patterns in human sera for rinderpest virus are now being investigated by Drs. W. Plowright and R. D. Ferris, to whom I am indebted for a resumé of their findings to date. Inactivated sera from 16 to 18 adults on the staff of a rinderpest research laboratory and, therefore, presumably exposed to this agent, neutralized rinderpest to titers between 0.6 and 1.9 log dilution. The two negative individuals were the only ones who gave no history of measles infection. Sera from a group of children taken during various stages of measles in a rinderpest-free area provided the following provocative data:

1 Nine of 17 developed rinderpest antibody during convalescence

2. Four children had antibody in both the acute and convalescent serum samples, but in each instance the first sample was obtained at least 5 days after the appearance of symptoms It may well have been that measles antibody was present as early as this after infection

3 Two sera remained negative after 5 weeks

4 One pair of sera with known measles antibody titers of 1.16 and 1:300 had levels for rinderpest of 0 and 1:4, respectively

While it is obviously premature to draw any sweeping conclusions from these findings, they are, nevertheless, in agreement with the results obtained in animal studies of canine distemper and rinderpest and consonant with the idea of one or more common antigens occurring in the viruses of measles and rinderpest

C. Immunologic Observations

The workers in East Africa have during the past few years contributed a series of intriguing observations on rinderpest virus that form the basis for postulation of a family which might include this cattle agent, measles, and canine distemper. When it was observed that distemper infection was rare in the dog population of a rinderpest-infested area, Polding and Simpson (1957) suggested a causal connection between these two events Rinderpest vaccine was then tested for its ability to protect dogs against canine distemper and was found capable of so doing This lead was pursued by Goret *et al* (1958), who inoculated ferrets with rinderpest virus and obtained complete protection against a challenge with virulent distemper Conversely, using ferret-origin distemper, they were able to protect calves against rinderpest, but with less virulent, avianized virus only 2 of 4 animals were found resistant to a challenge. Such a suggested interdependence between cross-protection and multiplication of the injected vaccine appears to have also been demonstrated by Polding *et al* (1959), who were unable to protect cattle with a canine distemper virus strain which did not cause viremia or clinical signs. Stated differently, it appears as though highly attenuated strains of either virus may be unsuitable for the investigation of cross-immunity studies because of their inability to multiply to a level sufficient to provoke an effective heterologous antibody titer

While investigating responses to vaccination with rinderpest Polding *et al.* (1959) found that in 2 dogs, a single 10-ml dose of rinderpest virus vaccine containing 30,000 ID_{50}/ml. of the virulent Kabete O strain failed

lenge with canine distemper virus In contrast to these findings is th observation that 20 ml of a rinderpest-immune serum failed to protec passively any of 5 dogs when they were exposed to distemper even thoug distemper-convalescent animals developed rinderpest antibody. Th parallelism between these immunologic findings and that existing betweer measles and distemper virus is striking. Again, we appear to be dealing with partial cross-protection wherein one member of a pair, in this case rinderpest, appears to be the "prime" antigen

By the application of the Ouchterlony technique, specific gel-diffusable antigens were demonstrable for rinderpest by White (1958) and for canine distemper by Mansi (1957). This technique has been adopted successfully by White *et al.* (1959) to obtain an *in vitro* cross-reaction between these two viruses. A single precipitate was observed when either rinderpest or canine distemper antigens were tested against homologous-immune antisera. Perfect coalescence of precipitate occurred when either sera was simultaneously run against both antigens. However, rinderpest serum appeared to have a somewhat greater reactivity with its homologous antigen These findings indicate that gel-diffusion methods may be of considerable usefulness in clarifying antigenic links between these viruses.

VI. Conclusion

It is now apparent to the reader that we are in a period of renewed interest in the viruses of measles, distemper, rinderpest, and those other agents, such as the so-called "foamy" viruses, which have in common a cytopathic effect in tissue culture consisting of syncytium formation and the production of intracytoplasmic and intranuclear inclusion bodies Although much recent work has been concentrated upon the elucidation of immunologic relationships, there is uncertainty as to the exact extent of antigenic overlap. This is not a matter of academic importance alone because the very real possibility exists that different members of this group, which are devoid of virulence for a heterologous host may, nevertheless, be employed for vaccination against a disease natural to this host. The successful demonstration of protection of dogs by rinderpest vaccine against distemper challenge furnishes a good example of this approach.

In Table VIII, we have attempted to illustrate graphically some of the known similarities and dissimilarities among members of the group. Large gaps in our information are apparent Additional definitive biophysical measurements would be helpful if they indicated particles of identical sizes so that the basis for inclusion of these three viruses in a common family could include similarity of physical properties. It

TABLE VIII

Reported Interrelationships of the Viruses of Measles, Distemper, and Rinderpest

Agent	Host		Susceptible cell culture	Immunologic crossing		Differences of possible significance
	Natural	Experimental		Serological	Protection	
Measles	Man (monkey)	*Dog*, suckling mouse hamster egg	Man, monkey, rodent, chick embryo			*Not infectious for ferret*
Canine *distemper*	Canines, several wild rodents, mammals	Ferret, suckling mouse, hamster, egg	Dog chick embryo			No clinical disease in monkey, CD antisera fails to fix complement with measles antigens
Rinderpest	Ruminant	Rabbit, suckling mouse, *hamster*, egg	Bovine, pig, *hamster*			No clinical disease in canines or (presumably) man

Note Established, *repeated observations* = ⟶ (thin solid arrow)
Preliminary observation = ⟶ (dashed arrow)
No reaction = ⟶ (heavy solid arrow)

would also be desirable to perform cross-immunologic tests in one laboratory where all three strains of virus could be studied simultaneously in a single host or tissue culture system. It appears to be only a question of time before this is accomplished

In conclusion, we have seen that alterations in the virulence of these three viruses are easily accomplished by serial transfers in alien host systems. Further, the antibody patterns that follow from infection with attenuated strains may be both quantitatively and qualitatively different from those that are produced by natural infection. For this reason, virulent fresh strains may prove to be the ones of choice for future work on antigenic sharing within this group.

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serum 12 to 24 hours after exposure has been claimed by Terragna (1959) who again focuses attention on the eye as a major portal of entry for measles

The combined experience of five groups engaged in collaborative studies of the Enders attenuated chick embryo tissue culture vaccines provides a sound basis for further extension of clinical trials of this live vaccine (*New Engl J Med* **263**, 153–180, 1960) Although reaction rates were appreciable, occurring in 142 of 171 children, their severity is considered to be of an acceptable order if we use as a reference the reaction to diphtheria-pertussis-tetanus, smallpox or typhoid vaccines encountered in pediatric practice Complete prophylactic efficacy was demonstrated in all persons who manifested an antibody response Impressive, although incomplete, protection was also reported by Hockenga *et al* (1960) using another CETC preparation

Current chick cell vaccines are of low infectivity when this live virus is administered intranasally That this may be due to their relatively low virus content is suggested by recent work of McCrumb and his associates (see Kress *et al*, 1960) who have studied a live vaccine grown in canine kidney tissue culture In this system infectivity titers are of a higher order, $10^{5.0}$ or greater $TCID_{50}$, than in avian cells In a small series of children, clinical reactions were reduced when the vaccine was administered intranasally, although antibody production and subsequent immunity to measles challenge compared favorably to that induced by the chick type vaccines inoculated parenterally We can anticipate further work on intranasal vaccination

Substantiation of the antigenic overlap of measles, canine distemper, and rinderpest viruses continues to appear, and differences in species response to these viruses is receiving more emphasis (Carlstrom, 1959, Imagawa *et al*, 1960, Mornet *et al*, 1960) Goret *et al* (1960) have shown that the canine distemper resistance induced in ferrets by rabbit-adapted rinderpest virus is due to an actual immunity and not interference Currently available attenuated canine distemper virus vaccines of egg origin are notoriously variable in potency, and the possible reasons for this are summarized in a good review article by Oxer (1960) Additional descriptions of the adaptation of canine distemper to cell culture systems have been reported by Vantsis (1959) and Hopper (1959) A preliminary report by Rockborn (1960) describes the further development of his dog kidney tissue culture vaccine which after 58 passages had a titer of $10^{6.5}$ $TCID_{50}$/ml Although highly attenuated, it conferred solid protection on dogs to challenge with virulent CDV Commercial development of such a vaccine is reputed to be under way

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Addendum

Several reports of a fundamental nature have appeared since this chapter was written In an effort to partially nullify the cliché that most reviews are out-of-date when they appear, these contributions are briefly discussed in the following addendum

A series of Japanese publications (summarized in Arakawa, Nagashima, and Kaneko, 1959) describe efforts to perfect inactivated measles vaccines using virus grown in mouse brain or chick embryo tissues Concentrated antigens were more effective than crude preparations in stimulating neutralizing antibody in a small series of humans Although protection against natural measles is claimed, the data is included in unsupported statement form only In the United States killed vaccines prepared in monkey kidney tissue culture (virus content not stated) were administered by Kempe (1960) to 47 children of presumptive seronegativity, CF of 1 4 or less, in 3 intramuscular doses of 1 ml each at 7-day intervals Only 17 developed CF or neutralizing antibody rises, although one is puzzled by the finding of relatively large numbers, 11 of 16, who had a CF rise without a concomitant elevation in neutralizing titer Unfortunately, an attempt to challenge the vaccinated group with live measles virus failed for technical reasons To date no well-documented reports of successful immunization with a killed measles vaccine have appeared Passive protection of children against measles by the conjunctival instillation of human convalescent

THE POLYOMA VIRUS

Section A

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I Introduction

A Tumor Induction with Cell-Free Extracts

In recent years considerable advances have been made in support of the virus theory of origin of cancer The search for virus-induced tumors was given new impetus when Gross (1951) described the induction of leukemia in strain C3H mice that were inoculated within 12 hours after birth with cell-free extracts prepared from the tissue of strain AKR mice with spontaneous lymphocytic leukemia Stewart (1953), while

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II PROPAGATION OF SE POLYOMA VIRUS

A. Source of Material

In the early experiments with the monkey kidney tissue cultures, attempts were made to recover polyoma virus from the following. (1) primary parotid gland tumors which arose in (C3H/Hen × AKR)F_1 hybrids that had received either cell-free extracts prepared from a transplanted AKR leukemia or supernatant fluids from monkey kidney cultures of the primary parotid gland tumors; (2) transplanted C3H/Hen leukemias, (3) spontaneous leukemias in strain AKR mice, and, (4) a transplanted paraganglioma that arose in a C3H/Hen mouse which had *been inoculated with a leukemia cell-free extract* With the mouse embryo cultures no attempts were made to recover the virus from spontaneous neoplasms Only the virus-induced tumors were tested

B Techniques

Materials to be tested for the presence of polyoma virus were prepared by one of three methods (1) the tumor was finely minced with scissors and a loop full of the mince added to the tissue cultures, (2) the tumor was homogenized with Alundum, centrifuged to throw down the coarse particles, and the tissue cultures were inoculated with the supernatant fluid, (3) cell-free extracts were prepared from the tumors as described previously (Stewart, 1955a) and were inoculated into the tissue culture Detailed descriptions of the techniques for preparing the tumor preparations and the tissue cultures are given elsewhere (Stewart *et al*, 1958a, Eddy *et al*, 1958a)

1 Passage in Mice

Stewart (1955c) described the propagation through 8 serial passages of a virus which produced tumors of the parotid glands and of the adrenal medulla Whole mouse embryos (strain C3H/Hen) 14 to 16 days old were passed through a 20-gage tumor press, mixed with filtered leukemia extracts (passed through a Morton glass filter of fine porosity), and 0 2 to 0 5 ml of the mixture was inoculated subcutaneously into 4–6-week-old strain C3H/Hen mice which had received 200 to 250 r total body radiation 1 to 2 hours earlier Twelve to fourteen days later mice with subcutaneous embryo growths were sacrificed and an extract was prepared from the liver, spleen, and kidneys * The extract was added to freshly

* Four of twelve mice which had subcutaneous embryo growths and were not sacrificed for virus passage developed tumors of the parotid glands 8 to 12 months after inoculation

attempting to repeat his work, failed to get leukemia in inoculated C3H/Hen mice, but reported tumors of the parotid glands in the mice 8 to 10 months after injection. Hybrid (C3Hf × AKR)F_1 mice which received filtrates made from similar cell-free extracts developed leukemias early in life. In further experiments with cell-free extracts prepared from leukemia tissues of either strain C3H/Hen or strain AKR mice, multiple types of neoplasms were observed in the inoculated mice (Stewart, 1955a,b; Stewart *et al.*, 1957). These were pleomorphic tumors of both major and minor salivary glands, adenomatous lesions of the renal convoluted tubules, renal sarcomas, mammary adenocarcinomas, epithelial thymomas, tumors of the adrenal medulla, and in the (C3Hf/Hen × AKR)F_1 hybrid mice, leukemias early in life.

Gross (1951, 1952, 1953a) reported first on the induction of leukemia in the mice which received cell-free extracts of spontaneous leukemias and later (1953b, 1955a) reported the occurrence of parotid gland tumors and sarcomas in mice receiving similar materials. He believed that there probably were three viruses, one for each type of neoplasm.

B. *Tumor Induction with Tissue Culture-Propagated Virus*

Conflicting results were reported on tumor induction with cell-free extracts. It is now recognized that an important factor in the lack of reproducibility was the comparatively small amount of virus in the preparations used. This difficulty was overcome when Stewart *et al.* (1957, 1958a) reported that the "pluripotent" oncogenic virus demonstrated in the leukemia cell-free extracts could be cultivated in tissue culture. With the virus grown in monkey kidney cells the spectrum of tumors observed in the inoculated mice was the same as that produced with highly potent cell-free extracts with the exception of leukemia. The cultures prepared from the minced mouse embryo were found more effective than the monkey kidney cells for the propagation of the tumor-producing virus as shown by: (1) an increase in the types of tumors induced in mice, (2) a greater frequency of certain primary tumors induced, (3) a shortening of the latent period or the time required for appearance of the tumors after inoculation; (4) successive passage of virus sublines (one has now been passed for 40 serial passages without loss of oncogenic activity), (5) tumor-inducing activity of the virus at dilutions of 10^{-7} and occasionally at 10^{-8}.

Because of its ability to produce a large variety of tumors Stewart and Eddy (1958a) proposed the name polyoma for the virus. In later publications it was designated as SE polyoma virus.

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1959). Henle *et al* (1959), in studying the development of polyoma virus in mouse embryo cells as revealed by fluorescent antibody staining, found that the nuclei of mouse embryo cells are affected first Thereafter virus-specific antigen gradually appears in the cytoplasm in increasing frequency and concentration and the nuclei lose the virus Those cells with virus in the cytoplasm generally showed signs of beginning degeneration

It was found that the tissue culture-propagated virus induced tumors, not only in highly inbred strains of mice, but that randomly bred Swiss mice were also susceptible to it (Stewart *et al* 1957, 1958a), and that it even produced tumors in other rodent species Tumors induced in this manner were reported in Syrian hamsters, Chinese hamsters, rabbits, and rats (Eddy *et al*, 1959a,b, Stewart, 1959, Stewart and Eddy, 1958b)

It is conceivable that in a continuous line culture of susceptible cells, if the cytopathogenic changes were controlled, an acceleration of cell growth or a hyperplasia resulting in neoplasms would occur Dawe *et al* (1959) maintained in tissue culture embryonic parotid gland tissue inoculated with polyoma virus and found that changes characteristic of parotid gland tumors were produced However, they failed to grow as tumors when implanted subcutaneously in mice

c *Mouse Lymphoma Cells* Rabson and Legallais (1959) reported the propagation of SE polyoma virus in milk-adapted murine lymphoma cells (strain P388D_1) A specific cytopathogenic effect was noted which persisted through continuous serial passages A pool of eleventh passage material titrated in the milk-adapted cells showed a titer of $10^{7.5}$ $TCID_{50}$

This work is of special interest as it demonstrates the replication of an oncogenic virus in malignant cells Recently, Banfield *et al* (1959) have described intranuclear and extranuclear particles in tissue cultures of P388D_1 inoculated with polyoma virus

C Recovery of Virus from Tumors

When monkey kidney cultures were used only 8 of 28 tumor preparations tested produced neoplasms when the supernatant fluids were inoculated into newborn mice With the mouse embryo cultures we have succeeded in recovering polyoma virus from 100% of the virus-induced tumors tested, whether they were from the mouse, hamster, rat, or rabbit.

III Anatomical Types of Tumors in Different Rodents

A Mouse Neoplasms

All strains of mice thus far tested have been found susceptible to tumor induction with SE polyoma virus if inoculated within a few hours after birth (Stewart *et al*, 1958a, Mirand *et al*, 1958) The tumor incidence

prepared embryo mash and used for the next passage. After 8 consecutive passages an oncogenic virus was still recoverable as evidenced by the development of tumors in mice which were inoculated shortly after birth with this material. Further passages were not made.

2 Tissue Culture

a Monkey Kidney Cultures. A search for an ideal system for propagation of the tumor-inducing virus was not made at this time. Because of their availability tissue cultures of monkey kidney cells were tried for the culture of the oncogenic agent (Stewart *et al*, 1957). Monkey kidney cultures maintained in medium 199 (Morgan, 1950), containing 1% calf serum, were inoculated with extracts or minces prepared from the tumors to be tested, and the cultures were incubated at 37°C. A fluid change was made after 1 week After 2 weeks' incubation 2 ml of fluid was passed to a fresh monkey kidney culture and part of the same fluid was tested for tumor induction in mice. Mice which received culture fluids after 1 to 3 passages in monkey kidney cells developed the same spectrum of tumors that were observed with an active leukemia cell-free extract

Good evidence of virus replication was not obtained in the monkey kidney cells, as it was not possible to carry the virus for more than 3 to 4 serial passages in these cultures because of simian viruses which destroyed the cells However, attempts to demonstrate virus in extracts made from the induced parotid gland tumors failed until after they were cultures on monkey kidney cells The monkey kidney cells may not have been a medium for virus replication but may have only released the virus from antibodies and other inhibitors

b. Mouse Embryo Cultures Tissue cultures made of minced mouse embryo cells were found to give excellent results from the propagation of polyoma virus (Stewart *et al*, 1958a, Eddy *et al.*, 1958a) The virus maintained its oncogenic activity after many tissue culture passages, one subline is still active after 40 serial passages Trypsinized monolayer mouse embryo cultures maintained in medium 199 with 1% calf serum were used in the early experiments With this maintenance medium specific virus cytopathogenic changes were not observed with regularity. This can now be attributed to an inhibitory substance in the calf serum, since undiluted calf serum mixed with virus was found to inhibit tumor induction in mice (Stewart and Eddy, 1958b) Two per cent horse serum substituted for the calf serum resulted in characteristic cytopathogenic changes in the cultures

Intranuclear viral inclusion bodies have been described in mouse embryo cultures of polyoma virus Intranuclear virus also has been visualized by electron microscopy in these cells (Dmochowski *et al*,

TABLE I

TYPES OF TUMORS AND INCIDENCE OBSERVED IN 400 SWISS MICE THAT DEVELOPED NEOPLASMS AFTER INJECTION WITH SE POLYOMA VIRUS*

Type of tumor	Percent of mice with specific tumor	Type of tumor	Percent of mice with specific tumor	Type of tumor	Percent of mice with specific tumor
Salivary glands and submucosal glands	96	Mesotheliomas of pleura and peritoneum	21	Adrenal medullary tumors	4
Renal lesions	48	Subcutaneous sarcomas and hemangioendotheliomas	16	Buccal mucosae carcinomas	3
Epithelial thymomas	26	Kidney sarcomas	15	Lung carcinomas	3
Mammary adenocarcinomas in both sexes	25	Epidermoid carcinomas	9	Lymphocytic leukemias	3
Bone tumors	23	Thyroid carcinomas	6	Visceral hemangiomas	2
Gastric carcinomas (epidermoid)	1	Sweat gland carcinomas	0.9		

* The tissues were examined histologically, many of them by Dr M F Stanton, National Cancer Institute, Bethesda, Maryland

in mice that were inoculated ranged from 80 to 100% with different culture preparations The latent period, or the interval from the time inoculated to the appearance of gross tumors, varied from an occasional mouse with neoplasms as early as 6 weeks to those that did not appear until 10 to 12 months after virus injection. The majority developed tumors in from 2 to 5 months

Many of the tumors were of types not known to occur spontaneously. Those observed and their frequency in over 400 virus-inoculated Swiss mice in which the tissues were examined histologically are shown in Table I A brief description of the tumors, all of which were reported previously (Stewart, 1955a, b, Stewart *et al*, 1957, 1958a), follows For a detailed description, see Stanton *et al* (1959a).

1 Pleomorphic Tumors of the Salivary Glands and Mucous Glands of the Head and Neck

a Incidence Tumors of the parotid glands occurred the most frequently Generally, 100% of the mice with neoplasms had this tumor. Only parotid gland tumors developed if the injected mice received a virus of low potency It thus appears that the parotid gland is the tissue most susceptible to the oncogenic effect of the virus With more active preparations the submaxillary and sublingual glands also developed tumors, in addition, the best virus cultures produced involvement of accessory mucous and serous glands of the head and neck

b Description of Tumors The parotid tumors were usually bilateral and multilobular In the small tumors the lobules were often about the same size but in the large tumors variation in size was found; frequently a lobule attained 2 to 2 5 cm in diameter The tumors generally were not adherent to the surrounding tissue and could be shelled out easily, but in several animals over 8 months old the tumors had become adherent to the overlying tissue and were infiltrated with a firm fibrous growth which extended throughout the subcutaneous tissue of the head and neck This is shown in Figs 1 and 2 Cysts filled with mucus were common in certain tumors

Histologically all the salivary gland and mucous gland tumors were the same and consisted of an epithelial component of tubules lined with cuboidal epithelium and of a fibroblastic component which infiltrated around the tubules The fibroblastic elements had many mitotic figures Many large lobules appeared to be primarily this tissue Lymphocytic infiltrations were frequent in areas of the tumors.

c. Metastases Pulmonary metastases usually confined to blood vessels were quite common and appeared to be independent of the size of the primary tumor or to the time it had been growing Metastases have

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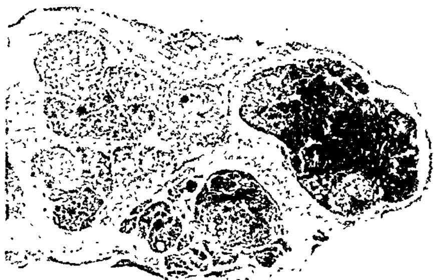


FIG 1 Parotid gland tumor in which the lobules are surrounded by sarcomatous tissue Magnification ×27

been seen in mice only 2 to 4 months old Figure 3 shows a 6-month-old Swiss mouse in which the lungs are heavily infiltrated with metastatic parotid gland tumor The parotid gland tumors were noted when the mouse was 3 5 months old

2. Renal Cortical Lesions and Renal Sarcomas

About one-half of the Swiss mice and 100% of the hybrid (C3Hf/Hen × AKR)F_1 mice which received tissue-cultured polyoma virus developed a lesion of the renal convoluted tubules Grossly, unless the kidneys also had sarcomas, the kidneys appeared normal except that often they were pale Histologically, the lesion varied from a few, scattered, enlarged, epithelial cells with ballooned nuclei as the only finding to marked involvement of the entire kidney cortex in which there were nests of dilated tubules with varying degrees of cellular hyperplasia In some, the hyperplasia resulted in a papillary infolding of the epithelium into the lumens resembling early adenocarcinomas The tubular

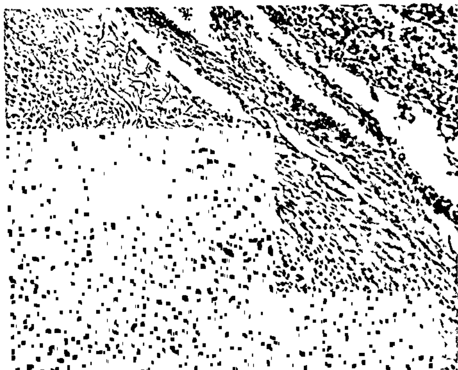


FIG 2 Area of tumor in Fig 1 showing the sarcomatous change of the subcutaneous tissue which had been in contact with the parotid tumor that is seen in lower right-hand corner Magnification ×125

lesion was always accompanied by infiltration of mononuclear inflammatory cells

About one-third of the Swiss mice with the renal cortical lesion also had kidney sarcomas which arose in the mesenchymal tissue of the pyramids Frequently several foci of early sarcoma were found in one kidney In several mice the tumors were bilateral A mouse with bilateral renal sarcomas is shown in Fig 4 As the sarcomas enlarged they caused blockage of the calyces, resulting in hydronephrosis

3 Epithelial Thymomas

This tumor developed in many of the virus-inoculated mice of different strains It was found in 26% of the Swiss mice but was probably more prevalent in the (C3Hf/Hen × AKR)F_1 hybrids It was in the hybrid that it was first observed and described (Stewart *et al*, 1957)



Fig 3 Six-month-old Swiss mouse with metastatic parotoid gland tumor

It varied in size from those visible only on microscopic examination to masses which almost filled the thoracic cavity They differed grossly from thymic lymphomas in that they generally did not invade the adjacent tissues but were circumscribed Some of the rapidly growing tumors in young mice were mucoid on cutting, in the others the consistency was very much like that of thymic lymphomas

Histologically, a thymus with a medullary tumor usually showed a marked decrease in both cortical and medullary lymphocytes This was pronounced even though the tumor was still very small As the tumor grew it replaced the normal thymic cells with an undifferentiated anaplastic cell In many of the early tumors the thymus showed a hyperplasia of Hassall's corpuscles In a few instances when the tumor



FIG 4 Four-month-old male Swiss mouse with bilateral kidney sarcomas Mouse also has a mammary adenocarcinoma, mesothelioma of the pleura and salivary gland tumors

occurred in very young mice, glandular tubular structures lined with cuboidal epithelium were observed mixed with the anaplastic cells A section through such a tumor is shown in Fig 5 The epithelial nature of the thymomas became very pronounced in tumor grafts made into irradiated (C3Hf/Hen × AKR)F_1 hybrid mice

4 Mammary Adenocarcinomas and Hair Follicle Epidermoid Carcinomas

The mammary tumors that occurred in 25% of the inoculated Swiss mice are of special interest because they developed in both males and females and because the architecture of the tumor is different from that

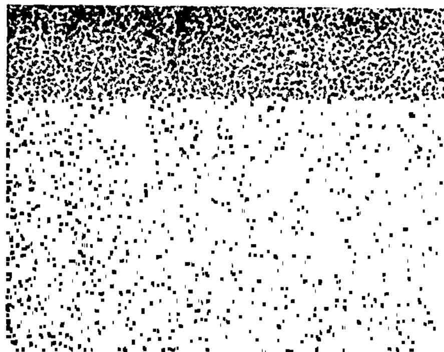


FIG 5 Section through an epithelial thymoma in a 2-month-old Swiss mouse Note glandularlike structures Magnification ×220

of spontaneous mammary tumors Also, they developed at a much earlier age than the spontaneous tumors and were often multiple Early tumors often developed in dwarfed mice 6 to 8 weeks old and frequently preceded the parotid gland tumors

About 40% of the mice with mammary carcinomas also had tumors of the hair follicles These papular lesions were most prominent over the ventral body surface, and sometimes extended onto the flanks or legs but seldom involved the back The papules were pale yellow, rarely ulcerated, and occurred in groups that became confluent. Histological sections revealed bizarre changes in the hair follicles, ranging from severe degeneration and atrophy through hyperplasia to the cytological characteristics of epidermoid carcinoma Metastasis to the lungs was observed from one of these tumors

5 Bone Tumors

Only a few bone tumors were observed in mice that received early tissue culture passages of polyoma virus, but many were seen in the mice that received virus after the twelfth passage in culture. Twenty-two

per cent of the Swiss mice inoculated developed bone tumors involving both membranous and long bones Tumors were common in the flat bones of the skull, in the mandibles, scapula, ribs, vertebrae, sacrum, innominate bone, and the long bones of the femur and humerus In the long bones the tumors were at the ends near the epiphyses Bone tumors were frequently multiple

The tumors ranged in diameter from those just visible to the unaided eye to 2 cm ; the larger ones were usually found in the rib cage, scapulae, or sacrum Those in the vertebrae and occasionally the skull generally caused some degree of paralysis, depending on the location Histologically, the tumors ranged from those benign in appearance in which the bone and cartilage cells showed advanced differentiation, to those which appeared very malignant These had scanty bone formation that was intermingled with a fibroblastic element in which mitotic figures were frequent Metastases to the liver and lungs were observed in several mice

6 Mesotheliomas

Twenty per cent of the inoculated Swiss mice developed these lesions, which were not observed in other strains of mice Usually they developed on the serous lining of the lungs, and occasionally the epicardium, pericardium, and peritoneum They could be seen with the unaided eye only when the process was so extensive as to render the serous surfaces translucent or opaque In some of the mice the mesotheliomas appeared as isolated tumors of the pleurae and measured 2 to 5 mm in diameter

The generalized lesions of the serous membranes were characterized by fingerlike projections of flattened cuboidal cells continuous with those of the normal mesothelial lining of the organs and were supported by a delicate framework of connective tissue These lesions were usually discrete, but in a few cases became confluent, producing nodular masses of acidophilic cells

7 Subcutaneous Sarcomas and Hemangioendotheliomas

The subcutaneous sarcomas were seen only in mice that lived for 6 months or longer In a few they were associated with the salivary gland neoplasms overlying and surrounding these tumors but in others they were independent of these or other tumors

The hemangioendotheliomas were generally in young mice and were one of the first lesions to appear These two tumors were similar to those that arise spontaneously or can be induced in mice with chemically defined carcinogens

8 Medullary Adrenal Carcinomas

This tumor which has its origin in the cell of the adrenal medulla occurred more frequently in strain C3H/Hen and (C3Hf/Hen × AKR)F_1 hybrid mice that had received leukemia cell-free extracts than in mice that received the tissue culture-propagated virus In one group of experiments with strain C3H mice, 36% developed this tumor (Stewart, 1955a) In the group of Swiss mice which received the tissue-cultured virus only 4% developed the adrenal tumor When only the advanced tumors that had broken through the adrenal capsule were examined histologically, origin in the adrenal medulla was questionable With the early tumors the origin is seen to be medullary. These tumors were usually very anaplastic and necrosis was frequent in the center of the larger masses

Invasion to the kidneys occurred in several of the mice and metastases to the liver, ovaries, and peripheral nodes was also common with the large tumors

9 Other Tumor Types

Thyroid Lesions Routine examination of the thyroid gland disclosed pathological changes in 6% of the Swiss mice Most of these consisted of enlargement of certain acinar cells with a ballooning of the nucleus Areas of normal-appearing thyroid tissues were mixed with *irregular acini containing little colloid and lined by small cells* interspersed with the enlarged cells, irregular cords of small cuboidal cells, and circumscribed, compact nodules up to 0 5 mm in diameter The nodules were made up of cords of larger cuboidal cells and distorted acini with some colloid Unquestionable carcinomas developed in a few mice *These were visible grossly and measured 2 mm or more.*

Epidermoid carcinomas of the buccal mucosae, visceral hemangiomas, squamous cell carcinomas of the stomach, sweat gland adenocarcinomas, and lung tumors These were among other tumors which it was felt could be attributed to the effect of polyoma virus

It is difficult to ascertain if any of the leukemias observed resulted from the virus inoculation With most virus preparations used, 100% of the injected mice died with early solid tumors With those virus preparations where there were late survivors a greater incidence of leukemias was observed than in controls of the same age The leukemia, however, *may have resulted from a nonspecific activation of a latent* leukemia agent This concept is supported by the fact that several Swiss mice that received polyoma virus which had been neutralized with antiserum and thus did not develop parotid gland tumors or other

tumors of the spectrum did develop acute lymphocytic leukemias (Stewart, 1959)

Nothing is known about the antigenic relationship between the various viruses reported which cause acute lymphocytic leukemia in mice (Gross, 1951, Schwartz *et al*, 1956, Moloney, 1959), or the relationship of these to polyoma virus Strain AKR mice with spontaneous leukemias that do not have polyoma virus antibodies have been reported (Rowe *et al*, 1959) Passive immunization of (C3Hf/Hen × AKR)F_1 hybrid mice with polyoma antiserum did not prevent the onset of leukemia (Stewart *et al*, 1959c). This, however, does not rule out a relationship, since we have shown that once polyoma virus enters the host cells the antiserum will not prevent development of tumors This probably also applies to the leukemia-inducing agent It is not known at what stage of development mice acquire the leukemia virus, Gross (1955b) has reported that it is egg-borne

Applying modifications of both Dulbecco's (1952) and Hsiung and Melnick's (1957) tissue culture plaque method to SE polyoma virus, we *have evidence indicating that one virus only is responsible for the* multiple types of neoplasms induced in mice, excluding leukemia (Stewart *et al*, 1959a) Eddy and Stewart (1959) reported that different lines of plaqued polyoma virus all have the same biological and physical characteristics and all induced the same types of tumors in hamsters

The possibility still exists, however, that polyoma virus produces only certain of the tumors observed in the inoculated mice and that it activates latent oncogenic viruses that are responsible for the others Since we have repeatedly recovered only polyoma virus from the different primary neoplasms it appears unlikely that other viruses are implicated

B Tumors in Hamsters

Eddy *et al* (1958a) were the first to show that Syrian hamsters inoculated when newborn were susceptible to tumor induction by SE polyoma virus At a later date Stewart *et al* (1958b) showed that hamsters through 24 days of age were also susceptible to tumor induction by the virus and that they developed the same types of tumors as when inoculated within a few hours after birth McCulloch *et al* (1959) confirmed tumor induction in hamsters with a polyoma virus subline recovered from a mammary tumor in a strain C3H/Jax mouse

Although the hamster virus-induced tumors appear at a much earlier date than the mouse tumors (some developed kidney sarcomas in 7 days), fewer tissues are affected The hamsters developed sarcomas and vascular tumors almost exclusively

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into newborn chicks it causes a rapidly fatal hemorrhagic disease involving the endothelial cells of the capillaries (Duran-Reynals, 1940) In older chickens it produces sarcomas

An interesting feature of polyoma virus, even though it is an oncogenic agent, is that in a susceptible host it produces both degenerative and hyperplastic lesions The earliest changes observed in an inoculated

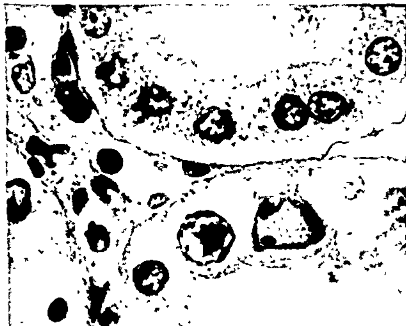


Fig 6 Section through kidney of mouse infected with SE polyoma virus Two cells of proximal tubule with enlarged nucleus, the one on the left showing a large inclusion body and the other degeneration The cells of the distal tubule appear normal Magnification ×1410 (Stanton *et al*, 1959a)

mouse are enlargement of certain epithelial cells with a ballooning of the nuclei These are most prevalent in the kidneys, but also are found in other organs (Stewart *et al*, 1959b, Stewart and Eddy, 1958a) Viral inclusion bodies are found in these cells (Stanton *et al*, 1959a) Figure 6 shows an epithelial cell of the proximal convulated tubules with a large inclusion body The cells in the distal tubules appear normal

The enlarged cells probably serve only for virus replication and cannot go into mitoses as the nucleus is destroyed Adjoining cells in which

1 Sarcomas and Angiomatous Tumors

The kidneys were the most common site for the sarcomas if the animals died early in life Hamsters 6 to 7 days old that were killed or died at this age had only this tumor, and in our experience the tumors were not grossly visible McCulloch *et al* (1959), however, have found the kidneys of 7-day-old hamsters tremendously enlarged due to a diffused sarcomatous process

As the hamsters aged, more areas became involved, 2- to 3-week-old animals developed sarcomas of the mediastinum involving the heart or the large vessels In many of these animals multiple kidney tumors could also be observed on gross examination, some also had small subcutaneous tumors Angiomatous tumors of the liver and lungs were very common Death frequently resulted from rupture of an angiomatous liver tumor Sarcomas of the organs of the gastrointestinal tract also developed with certain virus cultures These were seen on the serosal surfaces and in the mucosal surfaces extending into the lumens

For histological description of the tumors the reader is referred to the publication by Eddy *et al* (1958a)

C Tumors in Rabbits

The rabbit tumors were of interest because in this host they were nonmalignant and in all instances regressed within 1 to 4 months Rabbits inoculated within the first day of life developed multiple subcutaneous growths which closely resemble fibromas (Eddy *et al*, 1958a; Stewart and Eddy, 1958b)

D Tumors in Rats

The tumors in rats induced by SE polyoma virus, described by Eddy *et al* (1959b), are also of mesenchymal origin These were primarily renal sarcomas A 28% incidence was noted in 65 rats inoculated within the first day of life Although subcutaneous sarcomas were also seen they could not be attributed to the virus, since these are found as spontaneous tumors in rats of the colony

IV. Host Response to SE Polyoma Virus and to Tumors

A Early Changes Observed after Virus Inoculation

In all virus infections both degeneration and hyperplasia of the host cells occur The response seen following the inoculation with certain tumor viruses is governed by the state of sensitivity or susceptibility of the host An example is the Rous sarcoma virus. When inoculated

Large ballooned nuclei are common in the thyroid and renal epithelium at all stages in the development of the tumors They are also frequently observed in the bronchial epithelium and in the alveolar cells of inoculated mice It is of interest that in the thyroid and kidneys, where these enlarged epithelial cells are the most common, one rarely finds an unquestionably fully developed carcinoma, although one finds many early neoplastic changes

In the neoplasm of the salivary glands, adrenal medulla, and thymus, the characteristically enlarged epithelial cells are found only in the early stages of tumor development We were unsuccessful in demonstrating virus in cell-free extracts prepared from these tumors It was only by the tissue culture technique that virus was recovered. We do not intend to imply that the virus may finally disappear from the mouse neoplasms Polyoma virus was demonstrable in tumors induced by it even after grafts of tumor were carried, by repeated passages, for 7 years in mice from a virus-free colony (Stewart *et al*, 1960)

B Tumor Transplantation

1 Tumor Grafts in Mice

a Types Transplantable The following neoplasms, which occurred in strain C3H/Hen mice or in (C3Hf/Hen × AKR)F_1 hybrid mice, have been grafted to other mice and carried for a varying number of serial transplants tumors of the parotid glands, mammary glands, adrenal medulla, thymus, ovaries, and a paraganglioma With some of these neoplasms, in order for the tumor to retain its original histology, it was necessary to pretreat the mice by radiation Attempts were not made to transplant all the tumor types observed None which occurred in Swiss mice were transplantable because of the heterogeneity of this strain of mice

b Hypervolemia Because of an interesting observation in mice carrying grafts of the parotid gland tumors, these grafts will be described in some detail Attempts to transplant the salivary gland tumors that occurred in mice of inbred lines to untreated mice were not always successful However, if the mice were pretreated with 250 r total body radiation a few hours prior to making the graft, growth resulted from all tumor transplants in at least 1 of 4 or 6 injected animals The tumor grafts grew more readily on subsequent transplantation made into similarly irradiated mice These tumors retained their original histology through many passages as long as the host was pretreated by radiation, mice with such transplanted tumors developed hypervolemia and a marked leukemoid reaction Grossly, the mice had cardiomegaly,

neither inclusion bodies nor virus particles are demonstrable by cytochemical methods (Leuchtenberger *et al*), or by electron microscopy (Dmochowski *et al*, 1959) are those with active mitoses which develop

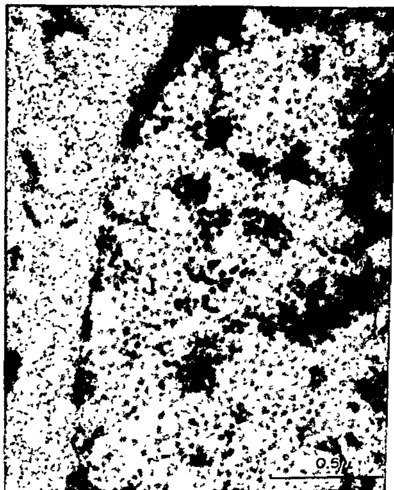


FIG 7 Portion of nucleus of mouse embryo cell in tissue culture infected with SE polyoma virus Virus particles 25–27 mμ diameter Magnification ×60,000 Courtesy of Dr Leon Dmochowski

into malignant cells. Figure 7 shows an electron micrograph of a mouse embryo cell with an enlarged nucleus in which the nuclear structures have been completely replaced by virus particles that measure 27 mμ.

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1 Tumor Grafts in Mice

a Types Transplantable The following neoplasms, which occurred in strain C3H/Hen mice or in (C3H/Hen × AKR)F_1 hybrid mice, have been grafted to other mice and carried for a varying number of serial transplants· tumors of the parotid glands, mammary glands, adrenal medulla, thymus, ovaries, and a paraganglioma With some of these neoplasms, in order for the tumor to retain its original histology, it was necessary to pretreat the mice by radiation Attempts were not made to transplant all the tumor types observed None which occurred in Swiss mice were transplantable because of the heterogeneity of this strain of mice

b Hypervolemia Because of an interesting observation in mice carrying grafts of the parotid gland tumors, these grafts will be described in some detail Attempts to transplant the salivary gland tumors that occurred in mice of inbred lines to untreated mice were not always successful However, if the mice were pretreated with 250 r total body radiation a few hours prior to making the graft, growth resulted from all tumor transplants in at least 1 of 4 or 6 injected animals The tumor grafts grew more readily on subsequent transplantation made into similarly irradiated mice These tumors retained their original histology through many passages as long as the host was pretreated by radiation, mice with such transplanted tumors developed hypervolemia and a marked leukemoid reaction Grossly, the mice had cardiomegaly,

neither inclusion bodies nor virus particles are demonstrable by cytochemical methods (Leuchtenberger *et al*), or by electron microscopy (Dmochowski *et al*, 1959) are those with active mitoses which develop

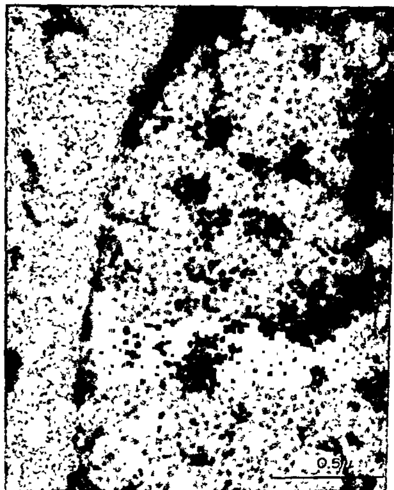


FIG 7. Portion of nucleus of mouse embryo cell in tissue culture infected with SE polyoma virus Virus particles 25–27 mμ diameter Magnification ×60,000 Courtesy of Dr Leon Dmochowski

into malignant cells Figure 7 shows an electron micrograph of a mouse embryo cell with an enlarged nucleus in which the nuclear structures have been *completely replaced by virus particles that measure* 27 mμ



FIG 9 Liver from strain C3H/Hen mouse carrying graft of sixth transplant generation of a parotid gland tumor Sinusoids of liver are greatly dilated and filled with blood Dark cells are leucocytes Magnification ×120

hypervolemia Nor was hypervolemia observed in irradiated mice which received grafts of the fibrosarcomas (transformed parotid gland tumors)

Hypervolemia was also observed in mice which had grafts of granulosa cell ovarian tumors that had occurred in strain C3H/Hen mice inoculated with leukemia cell-free extracts

c Serum Antibodies Mice with grafted tumors developed antibodies specific for polyoma virus Antibodies were demonstrable in high titer even after a transplanted virus-induced adrenal medullary tumor had been carried for 114 serial passages for a period of 7 years (Stewart *et al*, 1960)

2 Tumor Grafts in Hamsters

Both types of tumors, the angiomas and sarcomas, which are induced with polyoma virus, were found to grow when transplanted to untreated adult hamsters Each type of tumor retained its original histology Metastases to the regional nodes and to the lungs were noted in some of these (Stanton *et al*, 1959b)

hepatomegaly, splenomegaly, and hemorrhagic adrenal glands Microscopically, the sinusoids of the liver, spleen, and adrenals, and the glomeruli of the kidneys were engorged with blood This resulted in marked atrophy of the parenchymal cells of the liver, adrenals, and spleen Photomicrographs of these organs, in which the parenchymal cells were reduced to cords and the sinusoids were filled with erythrocytes and cells of the granular series, are shown in Figs. 8–10. Less marked

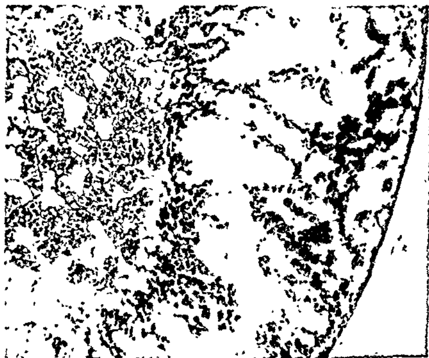


Fig 8 Adrenal gland from strain C3H/Hen mouse carrying graft of eighth transplant generation of a parotid gland tumor Cortex of gland has dilated sinusoids and atrophy of parenchymal cells Blood cells washed out by fixative. Magnification ×300

but similar histological findings were sometimes seen in the organs of mice with large primary parotid gland tumors

Blood volumes of mice with grafted tumors, recovered by decapitation of heparinized animals, ranged from 4 to 9 ml per mouse

Grafts of parotid gland neoplasms which were transplanted to non-irradiated mice lost their epithelial component after a few passages and appeared to be fibrosarcomas Mice with such grafts did not develop

The Bittner mammary tumor agent is probably the best example for the first group and the Shope papilloma and SE polyoma viruses are two that fall into the second group Only these will be considered as electron microscopy studies show an interesting difference in their origin within the cell. Because of this difference we wish to speculate on the possible connection between origin of the virus and the antigenic response of the parasitized host.

Bernhard (1958) and Moore *et al* (1958), in studying the mouse mammary tumor agent, found that the cell membrane of a parasitized cell participates in the formation of the viral capsule Microvilli formed in the cell membranes are pinched off with the virus particle or nucleoid as they are given off into the intercellular spaces This is shown in Fig 11 The virus particle thus supplied with an outer coat made of host protein cannot provoke an antigenic response in that same host The same virus inoculated into a foreign host would stimulate antibody formation The nucleoids of the virus measure 20 to 35 mμ and the whole virus 90 to 120 mμ Since the capsular membrane corresponds to the highly specific protein of the cell, this may also account for the fact that the induction of tumors is limited to mammary cells and that transmission of the virus is highly conditioned by the genetic constitution of the host

Electron microscopy of the Shope papilloma virus (Moore, 1959) and SE polyoma virus (Dmochowski *et al*, 1959, Bernhard *et al*, 1959) demonstrate an entirely different origin for these viruses from that of the mammary tumor agent Both of these viruses are exclusively intranuclear and are not encapsulated. The virus particles are 25–27 mμ in diameter, a size corresponding to that of the milk agent nucleoid. Since these viruses do not acquire a capsule from the host cell membranes they readily stimulate antibody formation in the parasitized animal Genetic constitution does not play a large part in determining susceptibility to these viruses

The natural host for the Shope papilloma virus is the wild cottontail rabbit, yet all rabbits are susceptible to it, and will develop papillomas when the virus is applied to the scarified skin Rabbits with tumors develop high titers of serum antibodies specific for the papilloma virus

The animals susceptible to the oncogenic effect of polyoma virus are of an even wider range As pointed out earlier, all strains of mice tested Syrian and Chinese hamsters, and also rats, develop malignant tumors when inoculated with this virus All rodents injected, whether or not tumors are induced, develop high titers of serum antibodies which can be demonstrated by tumor inhibition tests in mice and by the *in vitro* hemagglutination inhibition test

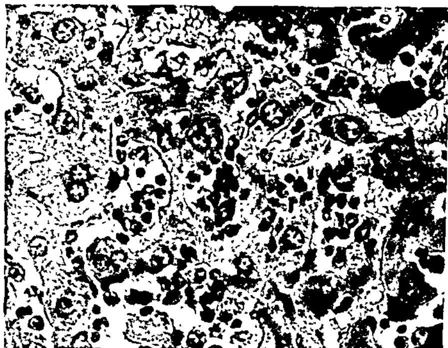


FIG 10 High power magnification of liver shown in Fig 8 showing marked leukemoid reaction Magnification ×1215

a Serum Antibodies Hamsters with virus-induced tumors were found to have serum antibodies to the virus (Eddy and Stewart, 1959) Antibodies were also observed in hamsters carrying tumor grafts after the first few passages, but after repeated passages they failed to show antibody titers by the hemagglutination inhibition method (Stanton *et al*, 1959b) Thus it seems that the virus may disappear from the transplanted hamster tumors

V ANTIGENIC PROPERTIES OF SE POLYOMA VIRUS

A *Antigenicity and Virus Origin*

Considerable variation has been reported on the antigenic response produced by the different tumor viruses Oncogenic viruses may be classified into two groups according to the antigenic response stimulated in the parasitized host· (1) those that evoke very little or no antibody response, (2) those that are antigenic and stimulate formation of antibodies

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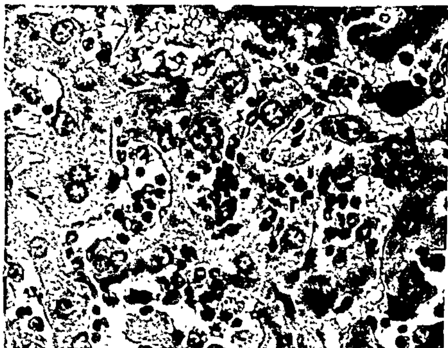


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B. Antibodies in Nontumor-Bearing Infected Animals

1. Occurrence in Laboratory Animals

Stewart *et al* (1959c, 1960) have shown that uninoculated adult mice housed in an environment with mice that have received polyoma virus acquire the infection; although they do not develop tumors, they develop antibodies which may be passed to the nursing offspring in the milk The offspring then become resistant to tumor induction by the virus Table II shows antibody titers in sera and milk from infected mice.

2 Occurrence Under Natural Conditions

Rowe *et al* (1959) have applied the hemagglutination inhibition test to studies on the prevalence of polyoma virus infections in laboratory mice and have found certain colonies which have a high incidence of infection

C Antibodies in Humans

Sera from cancer patients and from patients with other diseases were found free of polyoma virus antibodies when tested by the mouse protection test (Stewart *et al*, 1958a) and by complement fixation tests (Rowe *et al*, 1958)

D Passive and Active Immunization

Stewart *et al* (1959c) and Stewart and Eddy (1958a) have shown that sera from rabbits or mice containing polyoma virus antibodies can prevent the oncogenic activity of the virus by passively immunizing newborn mice with the serum or by mixing it with virus just prior to injection Tumor development could not be prevented when the serum was given one hour after the virus

Active immunization was also demonstrated (Stewart and Eddy, 1959) Hamsters were used as the test animal because they were found susceptible to tumor induction by polyoma virus through 24 days of age (Stewart *et al*, 1958b) Mice could not be used as those over 48 hours old were resistant except for an occasional animal that developed tumors For immunization studies it was necessary to use an animal that was susceptible to tumor induction in an older age group, otherwise nonimmunized control litter mates would be equally resistant to the virus challenge given the immunized animal

In preliminary studies, it was found that the virus was not completely inactivated when treated with 0 4% formalin and incubated 3 weeks at 37°C Two-day-old hamsters that received this formalized antigen



Fig 11 Milk agent mammary adenocarcinoma cell from RIII tumor taken into culture for 10 days showing microvilli with nucleoids along cell membrane and virus particles in intercellular space Magnification, ×40,120 Courtesy of Dr Dan H Moore

developed subcutaneous tumors in 3 to 9 months. Hamsters were accordingly immunized with a vaccine prepared from live virus mixed with antiserum. This was given in two doses, one when the hamsters were 2 days old, the other when they were 10 days old. The immunized hamsters and control nonimmunized litter mates were challenged with live virus at 3 weeks. Of 68 surviving hamsters which received the vaccine 85% failed to develop tumors after virus challenge, while 89% of 63 control litter mates, which received the same virus dose, developed tumors.

E. Antigenic Relationship to Other Viruses

Stewart *et al.* (1958a) have shown, by cross-neutralization tests with specific antiserum, that SE polyoma virus sublines recovered from strain AKR mice of the following sources were antigenically the same: (1) Dr. L. Gross's Laboratory, Veterans Administration Hospital, Bronx, New York, (2) Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, (3) Dr. L. W. Law's Laboratory, National Cancer Institute, Bethesda, Maryland.

No antigenic relationship was observed between polyoma virus and the following viruses: (1) Friend's leukemia virus, (2) Shope fibroma virus, (3) mouse K virus, (4) virus of lymphocytic choriomeningitis, and (5) the virus which causes bovine papillomas. A possible relationship with the last four viruses listed was considered for the following reasons: (1) The lesions produced in rabbits by the Shope fibroma virus and by the polyoma virus are very similar histologically; (2) the mouse K virus and polyoma virus both produce characteristic ballooned cells in the mouse lungs; (3) the virus of lymphocytic choriomeningitis was recovered from many of the transplanted mouse tumors from which tumor-inducing, cell-free extracts were produced (Stewart and Haas, 1956); (4) since bovine papillomas are very common in cattle and specific antibodies may be present in calf serum, the possibility existed that the inhibitory effect of calf serum for polyoma virus was due to bovine papilloma virus antibodies.

VI. Discussion

Although it has been demonstrated that SE polyoma virus produces tumors in rodents under laboratory conditions, it is known that the parotid gland tumors and others of the spectrum produced in mice rarely are found under natural conditions, even though it has been found that many mouse colonies have the virus as a latent infection (Rowe *et al.*, 1959). Introduction of large quantities of virus may be necessary to

TABLE II

ANTIBODIES IN MOUSE SERUM AND MILK AS DEMONSTRATED BY INHIBITION OF SE POLYOMA VIRUS HEMAGGLUTINATION OF GUINEA PIG ERYTHROCYTES*

Source of serum	Serum dilutions							
	1/80	1/320	1/1280	1/2560	1/5120	1/20,480	1/40,960	1/81,920
14-Month-old mouse inoculated with virus when 4 weeks old	–	–	–	–	–	–	–	±
14-Month-old mouse inoculated with virus when 4 weeks old	–	–	–	–	–	–	±	3+
14-Month-old mouse inoculated with virus when 4 weeks old	–	–	–	–	–	4+	4+	4+
21-Month-old mouse not inoculated, held in virus-contaminated quarters	–	–	–	–	–	4+	4+	4+
19-Month-old mouse not inoculated, held in virus-contaminated quarters	–	–	–	–	4+	4+	4+	4+
19-Month-old mouse not inoculated, held in virus-contaminated quarters	–	–	4+	4+	4+	4+	4+	4+
14-Month-old mouse not inoculated, held in virus-contaminated quarters	–	–	–	4+	4+	4+	4+	4+
9-Month-old mouse not inoculated, held in virus-contaminated quarters	–	–	–	4+	4+	4+	4+	4+
Source of milk	**Milk dilutions**							
	1/10	1/20	1/40	1/80	1/160	—	1/20,480	1/40,960	1/81,920
2 Mice, 5 months old, inoculated with virus shortly after birth	–	–	–	–	–		–	±	3+
2 Mice from virus-free environment	–	–	–	3+	4+		4+	4+	4+

* Minus signs indicate complete inhibition of hemagglutination. Plus signs preceded by numeral indicate degree of hemagglutination

Glossary

Adenocarcinoma A malignant growth made up of epithelial cells arranged in the form of glands

Angiomatous In the nature of tumor, the cells of which tend to form blood vessels or lymph vessels

Hemangioendothelioma A malignant growth of the endothelium of the capillary vessels

Hypervolemia Increased blood volume

Lymphoma A malignant neoplastic disease of the lymphoid tissue

Mesothelioma A tumor derived from the simple squamous-celled layer which covers the surface of serous membranes (the pleura, peritoneum, and pericardium)

Oncogenic Tumor-forming

Paraganglioma A tumor made up of cells resembling the chromaffin cells of the adrenal medulla, but occurring elsewhere than in the adrenals (the one referred to here occurred in the area of the parotid glands)

Thymoma A tumor derived from the epithelial elements of the thymus

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cause tumor induction which ordinarily does not occur in natural infections It is also possible that the virus is passed from mother to embryo *in utero* and that the mice acquire an immunological tolerance to the virus as in the case of *in utero* infections with the virus of lymphocytic choriomeningitis (LCM) in infected mouse colonies (Traub, 1935). Mice from such a colony carry large quantities of LCM virus but never show symptoms of disease unless it is precipitated by some outside stimulus (Traub, 1936) The experience of Stanton *et al* (1959a) in which they describe tumor induction in mice inoculated *in utero* with SE polyoma virus tends to discredit this concept for this particular virus Also against this idea is the fact that mice in an infected colony have antibodies to polyoma virus If they had acquired tolerance, antibodies would not be produced

The antigenic response provoked in the host by polyoma virus may account for the absence of spontaneous tumors in infected colonies Stewart *et al* (1960) have found that maternal antibodies passed to the nursing offspring in milk inhibit tumor induction

As is the case in inapparent infections with LCM virus, activation may be brought about with a given stimulus Carcinogens and even other viruses may produce tumors by the activation of latent polyoma virus infections. Radiation has been reported as activating a latent leukemia virus (Lieberman and Kaplan, 1959, Gross, 1959).

The mechanism by which an oncogenic virus produces tumors is purely speculative at this time, but this is also true of physical and chemical carcinogens With SE polyoma virus the following progression of events appear to occur in the development of the mouse tumors. The first change noted is a ballooning of some of the epithelial cells in certain organs This results from an enlargement of the nucleus which participates in the replication of the virus Because of the massive quantities of virus formed with the associated damage to the nucleus, these cells do not go into mitosis but are destroyed Adjacent cells or perhaps cells even in other organs are stimulated into hyperplasia and tumor formation. It is not known whether this is brought about by the virus directly or through the action of a by-product of the virus. But neither have we any knowledge of how hyperplasia is induced by vaccinia, measles, or herpes viruses When this is understood we may learn how such a progression of events can be prevented or stopped, once started.

In the event tumors in other mammals and in man may be virus-induced, it is possible that they go through a process in their development similar to that of polyoma virus-induced tumors. In such a case virus may be recoverable from other tumors only at the stage when it is actively replicating.

THE POLYOMA VIRUS

SECTION B

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I INTRODUCTION

The concept that tumors are induced by a microbiological entity has had occasional proponents over many years (Borrel, 1903, 1907, Boss, 1903, Gye, 1925, 1931, Gerlach, 1928, Andrewes, 1934) In spite of this cancer had been regarded for a long period of time as a disease apart from all others (Boycott, 1928, 1933, Ewing, 1940) The discovery by Ellermann and Bang in 1908 that leukemia in chickens could be transmitted by cell-free extracts was the first experimental evidence to support the theory of the viral nature of a neoplasm Other discoveries of tumor viruses followed the virus of chicken sarcoma by Rous (1911), human wart virus by Wile and Kingery (1919), infectious oral papillomatosis virus by De Monbreun and Goodpasture (1932), the rabbit fibroma and rabbit papilloma viruses by Shope (1932, 1933), the Bittner milk virus of mice (Bittner, 1936), and a frog tumor virus by Lucké (1938) These discoveries did not change the course of investigations of cancer in most laboratories Primary emphasis was directed toward determining the characteristics that distinguish cancer cells from normal cells (Graff, 1959) In 1951 Gross made the important observation that a cell-free leukemia extract would transmit leukemia to mice that were only a few days old This observation was confirmed by Stewart (1953), Woolley (1956), and Furth (1956) In most of these reports the virus or viruses were termed a "factor," "substance," "extract," "filterable agent," or "transmissible agent" Again, when an extract from mouse leukemic

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tion of either adenovirus or SE polyoma virus in tissue culture may increase over a period of 10 to 21 days The SE polyoma virus might be classed with other tumor-inducing viruses, such as Shope's rabbit (Shope, 1933) and deer (Shope *et al*, 1958) papillomas, the Rous chicken sarcoma (Rous, 1911), Friend's (Friend, 1957) and Gross's (Gross, 1951) leukemia viruses, Bittner's "milk agent" (Bittner, 1936), and others Except for the capacity to induce tissue proliferation it differs markedly from all of these viruses

II Size and Morphology

The size of a virus particle can be indirectly determined by a number of methods and directly by examination in the electron microscope In all these studies it is important that the material studied represent only the virus under consideration and that the results are correlated with biological activity Hamsters inoculated when <1 to 3 days of age with tissue culture-propagated virus filtered through gradocol membranes of 43, 77, 79, 82, 120, and 220 mμ pore size showed that all virus was retained by the 43 mμ pore size membrane and that most of it was retained by the 77 or 79 mμ membranes Only 2 of 51 hamsters injected with the filtrates or mouse embryo cell cultures that had been incubated with filtrates of virus passed through 77 or 79 mμ gradocol membranes developed tumors (Eddy *et al*, 1958c, Eddy and Stewart, 1958) Tumors developed in hamsters that received filtrates of the virus preparation or fluid of cell cultures incubated with filtrates that had been passed through the 82, 120, and 220 mμ membranes According to Elford's empirical formula the size range is one-third to one-half the pore diameter of a membrane just capable of retaining the virus Kahler *et al* (1959), using electron microscopy of partially purified virus in a pellet and zonal density gradient separation, obtained measurements of 44 mμ diameters for the virus particles The chromium-shadowed particles, like many other viruses, were spherical in shape The particle size of the virus was also studied by Bernhard *et al* (1959), but in ultrathin sections The particles identified as virus by these investigators were approximately 30 mμ in diameter

III Concentration and Purification

Concentration and partial purification of the virus has been accomplished by adsorption and elution from guinea pig erythrocytes, centrifugation in a number 40 rotor in a Spinco Model L centrifuge at 40,000 r p m (85,780–142,900 times gravity) for 3 hours, by alcohol precipitation in the cold (Eddy and Stewart, 1958) and more recently by drying *in vacuo* while frozen, redissolving the precipitate, and dialyzing against

tissue was propagated in monkey kidney (Stewart *et al*, 1957a), chick embryo (Stewart *et al*, 1957b), or mouse embryo tissue cultures, and when it was demonstrated to cross species lines and induce tumors in the hamster (Eddy *et al*, 1958a,b), it was referred to as a "substance released from tissue culture cells," and as an "agent" and "factor." In 1958 (Eddy *et al*, 1958d, Stewart and Eddy, 1959) it was first referred to as a virus and designated the SE polyoma virus. Since that time the virus has acquired other names: mouse parotid tumor virus (Kahler *et al*, 1959), mouse polyoma virus (Rowe *et al*, 1959), polyoma virus (Rabson and Legallais, 1958), and P or pluripotent virus (Buffett *et al*, 1958) These descriptive names are based upon pathological criteria As facts concerning the epidemiological aspects of infection with the virus become known, it is apparent that the induction of tumors under natural conditions represents only a fraction of the animals that contract the infection Animals kept in the same room with other infected animals develop immunity (Stewart and Eddy, 1959), but relatively few respond to infection with tumor formation (Eddy *et al*, 1959a, Rowe *et al*, 1958b)

From the viewpoint of the virologist, classification according to disease is only partially determined by the properties of the infectious virus, host factors play an impressive role It is well known that the host sometimes responds to entirely different etiological agents in the same way, *Rickettsia prowazeki* and *Salmonella typhi*, for example, may cause clinically similar disease The desirable goal is the complete identification of the virus by its physical and chemical properties In the case of the SE polyoma virus this characterization is impossible at present and reliance must be placed upon diagnosis by pathological and immunological criteria

It is generally recognized that viruses with similar characteristics fall into certain natural groups The SE polyoma virus does not seem to belong with any of the recognized natural groupings Henle *et al* (1959) have shown that it will interfere with the multiplication of vesicular stomatitis virus, but the interference phenomenon may occur between unrelated viruses Like herpes, lymphocytic choriomeningitis, or the K virus (Kilham and Murphy, 1953) it may remain latent or masked in the animal for long periods of time (Eddy *et al*, 1959a). It has been shown by the immunofluorescent antibody technique (Henle *et al*, 1959) to multiply first of all in the nucleus As infection progresses fluorescence is seen in the cytoplasm and eventually the virus is released from the cell There are certain similarities to the adenoviruses. The adenoviruses also multiply within the nucleus, and like the polyoma virus they may cause either proliferation or degeneration of cells. The concentra-

group consists of proteins or mucopolysaccharides which are relatively stable to heat but which are destroyed by the receptor-destroying enzyme (RDE) of *Vibrio cholerae* or by a combination of trypsin and periodate The second group are the lipid inhibitors and the third group are the heat-labile inhibitors The inhibitors are of great importance in choosing a serum to be incorporated in the nutrient medium for cell cultures Table I shows the effect of increased amounts of serum on the yield of

TABLE I
HEMAGGLUTINATION TITERS OF SE POLYOMA VIRUS IN FLUIDS FROM CELL CULTURES MAINTAINED WITH VARYING AMOUNTS OF SERUM

Serum lot	Virus strain	Serum	Virus titers
986	2609	20% Horse	1 20
		2% Horse	1 40
1793	2510	2% Calf	1 40
		1% Calf	1 160
820	2677	2% Calf	1 80
		1% Calf	1 160

virus Generally, horse serum appears to contain less inhibitors than calf serum and is used routinely in this laboratory as a constituent of the nutrient medium The following basic mediums have been successfully used lactalbumin hydrolysate (Melnick and Riordan, 1952), Eagle's (1955), and 199 (Morgan *et al*, 1950) A limited number of experiments carried out with medium 100 (Evans *et al*, 1959) without serum as a maintenance medium resulted in virus concentrations sufficient to induce tumors in hamsters Quantitative studies with this medium have not been carried out The fluids on all cultures are removed and replaced at weekly intervals

Four *in vitro* tests have been employed for determining the concentration of virus and antibody the cytopathogenic test (Eddy *et al*, 1958d), the hemagglutination or hemagglutination-inhibition test (Eddy *et al*, 1958c, Hartley and Rowe, 1959), the complement fixation test (Rowe *et al*, 1958a), and the mouse antibody production test (Rowe *et al*, 1959 The cytopathogenic test may be carried out by inoculating retrypsinized mouse embryo roller tube cultures with 10-fold dilutions of the virus and observing the cultures for cytopathogenic changes for a period of 2 or 3 weeks Cytopathogenic-inhibition tests are also carried out in roller tube cultures using as the inoculum either a series of 10-fold dilutions of virus plus antiserum to make a concentration of 1% or a series of 2-fold serum dilutions that have been incubated with approximately 1000 tissue culture infective doses at 37°C for 4 hours The

0.02 *M phosphate buffer solution pH 7.2 at 4°C.* (*Eddy, 1959*) To eliminate the possibility that more than one virus was responsible for the tumors produced in hamsters, the SE polyoma was grown in tissue cultures under an agar overlay and single plaques were removed, diluted, and new cell cultures were inoculated and overlayed with an agar medium Virus propagated from the second plaque passages induced neoplasms in the hamster (Eddy and Stewart, 1958) that were similar to the tumors induced by virus that had not been plaqued and some of the same fluids also induced an array of tumors in the mouse (Stewart *et al*, 1959a) Recently, Winocour and Sachs (1959) and Dulbecco and Freeman (1959) have also demonstrated that the virus may be recovered from plaques

IV In Vitro Tests

The SE polyoma virus was the first mammalian tumor-inducing virus that lent itself to propagation and titration outside the animal host It has been cultivated on a variety of tissue culture cells, mouse embryo (Eddy *et al*, 1958a), mouse kidney, mouse heart, mouse thymus (Eddy and Stewart, 1959), monkey kidney (Stewart *et al*, 1957a), and chick embryo (Stewart *et al*, 1957b) The mouse cells appear to be the cells of choice, although Rabson and Legallis (1958) report the growth of high titer virus on murine lymphoma cells (strain P388 D) Detailed nutritional studies of medium for the propagation of the SE polyoma virus have not been carried out but varying the amount of sodium bicarbonate in the medium (Eddy and Stewart, 1958) does influence the amount of virus growth Little cytopathogenic effect and low concentration of virus were obtained in cultures to which only 0 5 ml of 5% sodium bicarbonate per 100 ml of medium 199 (Morgan *et al*, 1950) was added In similar cultures to which 1 5 ml, 2 5 ml, and 3 5 ml of 5% sodium bicarbonate was added, increased cytopathogenic effects were noted and the hemagglutination titers were progressively higher Hsiung and Melnick (1957) had noted that the amount of sodium bicarbonate in the medium influenced the propagation of poliovirus in tissue cultures and more recently Chang (1959) has shown that bicarbonate participates in RNA and protein synthesis

A number of substances in serum and body fluids have been described which react with viruses and alter their biological activities The most important of these arise in response to previous exposure of the animal to the virus (Stewart and Eddy, 1959), but there are others, the so-called nonspecific inhibitors The nonspecific inhibitors fall into three general groups that are difficult to study because they occur singly or in varying combinations and amounts in different sera (Allen *et al*, 1958). One

group consists of proteins or mucopolysaccharides which are relatively stable to heat but which are destroyed by the receptor-destroying enzyme (RDE) of *Vibrio cholerae* or by a combination of trypsin and periodate. The second group are the lipid inhibitors and the third group are the heat-labile inhibitors. The inhibitors are of great importance in choosing a serum to be incorporated in the nutrient medium for cell cultures. Table I shows the effect of increased amounts of serum on the yield of

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986	2609	20% Horse	1:20
		2% Horse	1:40
1798	2510	2% Calf	1:40
		1% Calf	1:160
820	2677	2% Calf	1:80
		1% Calf	1:160

virus. Generally, horse serum appears to contain less inhibitors than calf serum and is used routinely in this laboratory as a constituent of the nutrient medium. The following basic mediums have been successfully used: lactalbumin hydrolysate (Melnick and Riordan, 1952), Eagle's (1955), and 199 (Morgan *et al.*, 1950). A limited number of experiments carried out with medium 109 (Evans *et al.*, 1959) without serum as a maintenance medium resulted in virus concentrations sufficient to induce tumors in hamsters. Quantitative studies with this medium have not been carried out. The fluids on all cultures are removed and replaced at weekly intervals.

Four *in vitro* tests have been employed for determining the concentration of virus and antibody: the cytopathogenic test (Eddy *et al.*, 1958d), the hemagglutination or hemagglutination-inhibition test (Eddy *et al.*, 1958e, Hartley and Rowe, 1959), the complement fixation test (Rowe *et al.*, 1958a), and the mouse antibody production test (Rowe *et al.*, 1959). The cytopathogenic test may be carried out by inoculating retrypsinized mouse embryo roller tube cultures with 10-fold dilutions of the virus and observing the cultures for cytopathogenic changes for a period of 2 or 3 weeks. Cytopathogenic-inhibition tests are also carried out in roller tube cultures using as the inoculum either a series of 10-fold dilutions of virus plus antiserum to make a concentration of 1% or a series of 2-fold serum dilutions that have been incubated with approximately 1000 tissue culture infective doses at 37°C for 4 hours. The

tubes are held for a period of 2 weeks and the medium containing the appropriate serum dilution is changed at the end of one week.

The adsorption of virus on erythrocytes occurs at refrigerator temperatures and is perhaps the quickest way to determine the concentration of the virus or its antibody The virus is heated to 37°C. for at least 2 hours or to 56°C for 30 minutes prior to the test. Dilutions are prepared in 2-fold steps in saline solution and an equal volume of 0.4% guinea pig cells are added, mixed, and the tubes are incubated at 4°C for approximately 2 hours or until the cells settle in a pattern on the bottom of the tube Hemagglutination-inhibition tests are carried out by combining 2-fold dilutions of serum with 4 hemagglutinating units of virus, incubating at 4°C for 4 to 18 hours, then mixing with 0.4% guinea pig erythrocytes, and incubating further at 4°C for 2 to 3 hours Titers are expressed as the highest serum dilution that prevents hemagglutination The virus is eluted from the erythrocytes readily at 37°C, and RDE is useful for removing some of the nonspecific inhibitors Guinea pig erythrocytes are generally used but cells from rats, mice, human O, sheep, dogs, chicks, and many other animals have also been agglutinated by the virus (Sachs *et al*, 1959)

Rowe *et al* (1958a) have reported that the complement fixation test may be used to determine antibody in sera The test has not been used as widely as the hemagglutination-inhibition test for the determination of antibody

The mouse immunity production test (MAP) (Rowe *et al*, 1959) has been used extensively by Rowe and his co-workers in epidemiological studies Adult mice are injected with material suspected of containing virus Two weeks later the mice are bled and their sera are tested for inhibition of hemagglutination The test is sensitive but, because of the danger of aerial infection with the virus, complete isolation of the injected animals is imperative and control animals must be maintained and tested

V Resistance to Physical and Chemical Agents

Preliminary studies indicate that infectious SE polyoma virus can be produced by mouse embryo cells exposed to viral nucleic acid or viral nucleic acid treated with the crystalline* enzyme ribonuclease at room temperature for 15 minutes but not with viral nucleic acid treated with the crystalline† enzyme deoxyribonuclease at room temperature for 15 minutes (Di Mayorca *et al*, 1959). Cytopathogenic changes due to the virus derived from nucleic acid occurred in mouse embryo cell cultures

* Obtained from the Sigma Chemical Co, 3500 DeKalb St, St Louis, Missouri
† Obtained from the Worthington Biochemical Sales Co, Freehold, New Jersey.

and fluids from the cultures induced neoplasms in hamsters A typical experiment is shown in Table II

Although knowledge of the chemical nature of the virus is rudimentary, some of its physical and chemical properties have been studied The data concerning these properties are of value for classification purposes, others bear on the practical problems of preservation of the virus, or prevention of dissemination of the virus in the laboratory or in breeding colonies of laboratory animals The virus is remarkedly resistant to physical and chemical agents that destroy many microorganisms and this is probably a factor in its tendency to persist in the laboratory environment Heating the tissue culture-propagated virus to 60°C for 30 minutes caused no loss in activity as determined by tumor induction in hamsters Hamsters injected with virus heated to 60°C for 60 minutes developed tumors slowly, and 4 of 19 hamsters injected with virus heated to 70°C for 30 minutes had developed tumors after 157 days (Eddy and Stewart, 1958, Eddy *et al*, 1958e)

The tumor-inducing capacity of the virus is not destroyed by prolonged periods of storage Neoplasms in hamsters have been induced by virus kept at −70°C for as long as 404 days, at −20°C for 329 days, and at 4°C for 169 days Anesthetic ether has no effect on the virus and this property serves to differentiate the virus from the myxoviruses and the ether-sensitive encephalitis viruses It may also serve to free tissue culture-propagated virus from bacterial contamination. An equal volume of ether is added to the contaminated culture, the flask or bottle is closed with a cotton stopper, and the culture is held at room temperature until the ether evaporates Trypsin, 0 25% in Earle's (1943) solution, does not affect the virus The virus has also been isolated from tumor tissue from a hamster stored in 33% glycerol for 72 days at 4°C

Formalin in 1 4000 concentration has been used to prepare vaccines against the SE polyoma virus but no systematic study of the action of formaldehyde on the virus has been carried out The virus in one vaccine, prepared by adding formalin to freshly harvested virus (Stewart and Eddy, 1959), was not all killed as evidenced by the induction of tumors in hamsters, the other (Eddy *et al*, 1959a), made by adding formalin to viral material that had been stored for several weeks at −20°C, did not induce tumors in hamsters, but blind passages of the material were not carried out It is well known that some of the formalin may be bound by impurities thus reducing the concentration of formaldehyde that is free to combine with the virus

Antibiotics, such as penicillin or streptomycin sulfate, are used routinely in tissue culture medium but no effect on the virus has been noted.

TABLE II

INFECTIVITY OF SE POLYOMA VIRUS NUCLEIC ACID AND SE POLYOMA VIRUS FOR MOUSE EMBRYO CELL CULTURES AND HAMSTERS

	Degenerative changes in tissue culture*											Hamsters			
	1st passage (days)							2nd passage days							
	7	14	21	28	35	42	49	7	14	21	28	Number injected	Number lost	Number with tumors	Period of observation (days)
Virus	1	4						0	2	3					
Virus combined with DNAase	1	4						0	3	3					
Virus combined with RNAase	1	4						0	2	3					
Nucleic acid	1*	1	3					3	4			10	3	7	18
Nucleic acid held at 20°C for 15 min	1														
Nucleic acid combined with DNAase	0	0	0*	0	0	0	0	0	0	0	0	15	0	0	88
Nucleic acid combined with RNAase	±	±	3*					3	3	4		13	7	6	22

* Cytopathogenic changes scored 4 (complete degeneration of cells) to 0 (no degeneration)

* Fluid from these cultures were injected into hamsters <1 to 3 days of age

VI Infectivity for Animals

Like many other viruses, the SE polyoma is capable of infecting different animal species Mice (Stewart *et al*, 1958a), hamsters (Eddy *et al*, 1958a), rats (Eddy *et al*, 1959b), and guinea pigs (Eddy *et al*, 1960) injected when newborn develop malignant neoplasms In the rabbit (Eddy *et al*, 1959c) multiple, small, subcutaneous nodules develop that later regress The virus may be recovered from tumor tissue from any of these animals by cultivation in mouse embryo tissue cultures The virus has been passed from mouse to mouse or to hamster, from hamster to hamster, mouse, or rabbit, from mouse to rat to hamster (Eddy and Stewart, 1958), and from rabbit to rabbit or to hamster with intervening tissue culture passage The tumors that are induced are characteristic for the host and do not appear to be affected by passage of the virus in another species Virus from a parotid gland tumor in a mouse, induced a renal tumor in a rat, and virus from the rat caused the development of tumors of the heart, lungs, liver, stomach, and intestines in a hamster Transfer of the infectious tumor from animal to animal, except with cells of compatible animals, is difficult, apparently because of the presence of specific antibody in the tissues of the tumor-bearing animal (Eddy and Stewart, 1958) Humoral antibodies do not prevent the transplantation of a virus-induced tumor from one hamster to another (Habel and Atanesiu, 1959)

VII Summary

Except for the capacity to induce tumors, the SE polyoma virus has characteristics that are typical of many of the well-known viruses It can be freed from other viruses by the plaque technique, concentrated, and at least partially purified by methods that have been useful for other viruses, namely, adsorption and elution on erythrocytes, centrifugation, alcohol precipitation in the cold, and by a combination of drying and dialysis Virus or its specific antibody can be measured by *in vitro* tests, such as cytopathogenic changes in roller tube tissue cultures, hemagglutination, complement fixation, or mouse antibody production Virus production in mouse embryo tissue cells with nucleic acids derived from the SE polyoma virus or nucleic acid treated with the enzyme ribonuclease has been demonstrated No virus production has been detected in preliminary experiments with viral nucleic acid treated with deoxyribonuclease The virus is resistant to many physical and chemical agents that are deleterious to other microorganisms, such as heating at 60°C for 1 hour, trypsin, ether, glycerol, desiccation, or prolonged periods of

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Virus combined with RNAase	1	4						0	2	3					
Nucleic acid	1[a]	1	3					3	4			10	3	7	18
Nucleic acid held at 20°C for 15 min	1														
Nucleic acid combined with DNAase	0	0	0[a]	0	0	0	0	0	0	0	0	15	0	0	88
Nucleic acid combined with RNAase	±	±	3[a]					3	3	4		13	7	6	22

* Cytopathogenic changes scored 4 (complete degeneration of cells) to 0 (no degeneration)

[a] Fluid from these cultures were injected into hamsters <1 to 3 days of age.

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storage at $-4°$, $-20°$, or $-70°C$ It is infectious for mice, hamsters, rats, rabbits, and guinea pigs The virus can be recovered from tumor tissue from these animals in mouse embryo tissue cultures

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THE FIBROMA-MYXOMA VIRUS TRANSFORMATION

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> For my part, I have resolved, in my search after knowledge of things, perseveringly to follow such an order as will require that I begin always with things which are simplest and easiest
>
> DESCARTES

I INTRODUCTION

The present chapter relates how tissue culture, by enabling one to perform the transformation of fibroma into myxoma virus in a predictable fashion, has opened up prospects of investigating the behavior of

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ates Once the transfer has been made, however, and the segment bearing streptomycin resistance has become incorporated by a process of recombination, the result is a stable clone which perpetuates the trait to succeeding generations Many different traits can be transferred by this method In the opinion of Ephrussi-Taylor (1959), DNA-induced transformations among pneumococci are now an extremely effective system which will continue to contribute to our understanding of DNA activities within cells

B Pneumococcal Transformation and the Berry-Dedrick Phenomenon

Animal viruses have contributed little basic information on genetic transfers mediated by DNA The reasons are not far to seek These viruses multiply in animal cells which are complicated systems to handle in comparison with bacterial cells growing in artificial media. Much, however, can be found out by means of tissue culture experiments An immediate question is whether fibroma-myxoma virus transformations are really transformations at all Do they represent some process other than genetic transfer? The logical place to start such an injury is the historical development of the Berry-Dedrick phenomenon in relation to work on the transformation of pneumococcal types

Griffith (1928) started what was to be the DNA-chain reaction of research with an accidental observation He was investigating serological reactions of pneumococci, a subject of therapeutic importance at the time, when he happened to vaccinate mice with heat-killed, virulent, Type III organisms The vaccine alone had no obvious effect on the animals When the mice were challenged, however, with rough, avirulent, Type II organisms, which had little effect by themselves, a remarkable change took place Many of the mice subsequently died with living, encapsulated, Type III pneumococci in their blood streams Berry and Dedrick (1936a,b), influenced by Griffith's findings, recognized the possible parallel between fibroma and myxoma viruses in the rabbit and the avirulent and virulent strains of pneumococci in the mouse Their experiments confirmed this parallelism Thus when rabbits were inoculated with live fibroma, which is a relatively benign virus, along with the highly virulent myxoma that had been killed by heat, the animals died within a few weeks of myxomatosis The transforming agent myxoma (TAM) was relatively heat-stable It remained active in transformation after 30-minute exposures to 60 or to 70°C but lost its activity at 90°C (Berry, 1937) Several features of Berry and Dedrick's work appear remarkable in the light of later developments One was that so many of their rabbit experiments were successful and another was that they were able to induce transformation with a wide latitude of tech-

the deoxyribonucleic acids (DNA) of the viruses involved and their relation to processes of infection in mammalian host cells. This approach to the Berry-Dedrick phenomenon (1936a) is a relatively new one It will be of value, nonetheless, to consider it in the broad setting of what is known of DNA in other microbial systems in order to gain perspective before consideration of actual experiments

A DNA in Microbial Systems

A stimulating aspect of Avery and associates' (1944) discovery that DNA was the substance bearing genetic information in transformation of pneumococcal types was that it furnished a unifying principle relating a specialized field to the study of heredity among all other types of living organisms Genetic information is carried by ribonucleic acid (RNA) among many viruses In others, however, which range from bacteriophages to higher animals, the unit bearers of heredity consist of DNA Many of these data have been learned in experiments involving transfers of genetic material and, since the field is large, it may be well to clarify a few of the terms and simpler mechanisms involved A typical bacteriophage consists of a central mass of DNA surrounded by protein. This protein coat functions in adsorption of the phage to the wall of its host bacterium and in injection of the more or less pure DNA into the bacterial cell All subsequent multiplication of virus, replication of its protein coat, and final lysis of the bacterium stems from the activity of the entering nucleic acid In one sense the invading DNA installs a new machinery within its host cell (Brenner, 1959) Some bacteriophages do otherwise than to infect and destroy their hosts In a process of transduction, phages may transmit genetic segments from one bacterium to another The fact that only a single trait is transferred is of importance It indicates that transduction is different from bacterial conjugation, which is a sexual union involving transfer of the whole genome of a male cell into a differentiated female cell (Jacob and Wollman, 1959) Partial genetic transfer can also take place among strains of bacteria which harbor no lysogenic strains of bacteriophage. This phenomenon is illustrated by the classic transformations among pneumococci As outlined by Ephrussi-Taylor (1959), the transfer usually involves only a single property, such as streptomycin resistance, which occupies only a segment of the total strand of DNA possessed by the donor pneumococcus It appears, for example, that this donor DNA breaks it up into small units in the process of extraction These molecular units are the ones taken by recipient strains and incorporated into their own genetic mechanisms The receptivity of the recipient cells for the foreign DNA is of short duration It may last for only 15 min-

reasons One is historical Transformation is the original term and parallels were early apparent between investigations on pneumococci and the Berry-Dedrick phenomenon Another reason is that a great deal more investigation will be needed before one can elucidate reactions between TAM and the fibroma virus The finding that DNA is involved in the reaction is one step in that direction. It is hoped that if one can further purify the transforming agent, analyze the intracellular conditions involved, and develop more delicate methods for recognizing the presence or absence of genetic change, it may be possible to define the nature of what is taking place Advances made up to the present time are discussed in the following account

II Nature of the Viruses Involved

It has become increasingly apparent that the characteristics of poxviruses, which include fibroma and myxoma, are of basic importance to the studies on their viral DNA in relation to host cells Some characteristics of these viruses are as follows:

A Diseases Produced in Rabbits

The essential factor in performance of transformation experiments is the wide difference in pathogenic effects produced by two closely related rabbit viruses Thus fibroma virus leads to a benign, localized tumor which regresses within a week Myxoma virus, on the other hand, leads to one of the most fatal diseases known among animals, nearly 100% of rabbits inoculated with it succumb to an overwhelming, systemic infection within 10 to 14 days The course of this disease is of interest It begins with a local tumor at the site of inoculation, but within 5 days the virus has begun to invade the body of the rabbit, as indicated by accumulation of white pus at the corners of the eyes, blotchy ears, and a steady downhill course This disease picture gives a clear end point to transformation experiments So far I have not found any tissue culture system which has provided an equally reliable end point, although development of such a test, by eliminating the inconvenience of using rabbits, would have considerable value

Some few rabbits used for testing have presented a picture intermediate between the two disases described above I have called this condition fibromyxoma (Kilham, 1958) The afflicted rabbits develop discrete, metastatic tumors on ears, eyelids, and tips of noses, among other situations, but their eyes remain clear and the animals usually survive for 3 weeks, if not indefinitely A first impression was that this disease was due to a virus intermediate between fibroma and myxoma, such as might result from a partial transformation This impression has not

niques, such that live virus and TAM did not have to be mixed prior to inoculation and that it was not necessary even to inject them into the same sites on the rabbit These investigations were never published in full The only accounts were four brief notes, but the phenomenon was confirmed by others on repeated occasions Results, however, have been irregular Hurst (1937) appeared to have little difficulty in effecting transformations, Gardner and Hyde (1942) and Smith (1952), on the other hand, stated that it was difficult or impossible to obtain regular results and other investigators, such as Meek and Acree (1939) were unable to demonstrate transformation at all

Investigations of transformations among pneumococci, by way of contrast, have developed in a more consistent fashion. Performance of experiments *in vitro* instead of *in vivo* and identification of the transforming agent as DNA (Avery *et al*, 1944) were stages of special significance A subsequent development has been the demonstration of an exchange of genetic units The degree to which this is possible is illustrated by the work of Bracco *et al* (1957)

Work done on the Berry-Dedrick phenomenon up to the present time is roughly comparable to the earlier stages of the investigations on the pneumococci I have, for example, substituted *in vitro* for *in vivo* experiments with a demonstration that fibroma-myxoma transformations can be effected in tissue culture in a predictable fashion (Kilham, 1957, 1958) These experiments were performed with heat-killed myxoma Some of the properties of this HEAT-TAM have been described by Kilham *et al* (1958), a following step was to demonstrate that myxoma virus DNA was the active constituent of the HEAT-TAM (Shack and Kilham, 1959).

C Is the Berry-Dedrick Phenomenon a Transformation?

There is no real evidence that the transformation of fibroma into myxoma virus involves an exchange of genetic units any more than this event was obvious in early stages of the pneumococcal investigations One can only speculate as to what actually takes place It was Berry's (1937) opinion that something in the TAM lent virulence to the fibroma virus and Gardner and Hyde (1942) stated that "it may be that the fibroma virus reactivates the heat-inactivated myxoma virus, perhaps by supplying an inactive component" A somewhat similar theory was discussed by Kilham in 1958 Fenner *et al* (1959), however, have recently expressed the idea in a more definite fashion by terming the Berry-Dedrick phenomenon a "reactivation," pointing out that bacterial transformation differs in having DNA as the active material I have preferred, however, to retain the term "transformation" for a variety of

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been substantiated In the first place, passage of a fibromyxoma tumor to another rabbit results in typical myxomatosis and, second, one can reproduce the full picture of the intermediate disease either by inoculating myxoma 2 to 3 days after fibroma or by inoculating a mixture containing a large fraction of fibroma and a small fraction of myxoma virus

These findings in regard to fibromyxoma are difficult to reconcile with ideas advanced by Smith (1952), who believed that the transformation of fibroma to myxoma virus might take place in a "stepwise" fashion analogous to that described for transformations among pneumococci by MacLeod and Krauss (1947) Smith apparently based her ideas on two findings One was the histological appearance of tumors induced and the other the fact that two rabbit passages might be needed before full myxomatosis appeared It is possible, however, for a small amount of myxoma to be obscured by a large amount of fibroma virus If this situation had obtained in Smith's experiments, fully formed myxoma virus could have been present from the start but would not have produced its full effects until a second passage Smith and also Berry (1940) considered that fibroma and myxoma included a "spectrum" of viruses Such a series would pass from the purely inflammatory IA strain to the original OA and Boerlage strains of fibroma virus, to neuromyxoma, and fully virulent myxoma viruses It is possible that each of these strains can be enhanced by the one above it in the "spectrum"

B Virulence and Thermoresistance

Myxoma and fibroma are both members of the family of poxviruses (Fenner and Burnet, 1957) and one wonders on what factors the great virulence of one as compared with the other of these two agents may depend Thermoresistance may be one such factor Thus, while fibroma virus grows only in parts of the rabbit which have a lower temperature, such as the skin, subcutaneous tissue, and testicle, myxoma virus, with a capacity to grow at a slightly higher temperature, is able to proliferate within the body and to cause death following systemic invasion. This theory is not altogether new. Thompson (1938), in attempts to grow the two viruses in rabbits maintained at elevated temperatures, found that fibroma virus grew poorly in animals exposed to a temperature of 34°–36°C and that other rabbits kept at 36°–40°C were able to survive infections with myxoma virus These experiments did not provide an adequate answer to the question of virulence It is difficult to believe that rabbits can maintain normal health when living in such abnormally high temperatures and poor health alone, as one can see by the effects of intercurrent infections in test animals, can depress the growth

of both fibroma and myxoma viruses *In vitro methods* seemingly offer a more precise way of determining thermoresistance. In one set of experiments (Kilham, 1959) fibroma and myxoma viruses were propagated in cultures of rabbit kidney incubated at different temperatures, ranging from 30° to 40°C Infectivity titrations were performed in rabbit skin on fluids and cells from these cultures 3 to 7 days after inoculation It was apparent from the results of these tests that fibroma virus grew to a lower titer than myxoma even under an optimal temperature of 36°C, while it grew with difficulty at 38°C, no proliferation could be induced or detected at 40°C Myxoma virus, on the other hand, could be maintained in repeated passages at 40°C, which is close to a rabbit's body temperature Cytopathogenic effects (CPE) gave further indication of this higher thermoresistance Myxoma induced a marked CPE in rabbit kidney cells at 40°C, whereas fibroma virus produced no effect The fibroma cultures were indistinguishable from uninoculated controls

Thompson and Coates (1942) obtained somewhat similar results in temperature experiments They found that myxoma virus would proliferate to a low titer in chick embryo cultures kept at 40°C but not in those kept at 42°C Unfortunately, they made no comparative studies of fibroma virus From our own experiments it appears that myxoma virus grows faster, to a higher titer, and under higher degrees of temperature than its less virulent relative Lwoff (1959) has noted a similar correlation of thermoresistance and speed of multiplication in relation to the virulence of poliovirus These points are of interest to transformation experiments, for they indicate that a small amount of myxoma, if it appeared, might outgrow and replace a larger initial amount of fibroma virus

C Virus Growth in Tissue Culture

Transformation takes place in tissue culture and some conception of virus growth, which may facilitate an understanding of processes involved, can be obtained by following the effects of the two agents on rabbit kidney cells Fibroma and myxoma induce a similar CPE This consists of a rounding of cells which appears 3 to 5 days after inoculation of myxoma and a few days later in the case of fibroma virus Formation of inclusion bodies can be followed to best advantage in cultures of cottontail rabbit testes, where fibroma virus grows in scattered areas with zones of normal cells in between (Kilham, 1956) The infected areas have two features in cultures stained a week after inoculation One is the presence of dense, acidophilic inclusions, located close to the nucleus and with a clear zone like a halo (Fig 1) and the other of cytoplasmic vacuoles containing a ground-glasslike substance, which



FIG 1 Intracytoplasmic inclusions in tissue culture of cottontail testes inoculated with fibroma virus (H & E × 1000)

may be a metabolic by-product of virus growth Myxoma produces similar inclusions in cultures of rabbit testes The myxoma bodies, however, are more widely scattered and irregularly shaped than those of fibroma virus Since these inclusions are the sites of virus replication and probably of transformation as well, their structures as revealed by electron microscopy are of interest

D The Natural History of Poxviruses as Revealed by Electron Microscopy

It is not known how poxviruses attach to and enter susceptible cells This may be a critical period in which the virus loses its mature form

and redirects cell physiology to its own purposes Less speculation is needed for events after this initial period Electron microscope studies have revealed that poxviruses all follow much the same course and the stages of development described by Gaylord and Melnick (1953) for vaccinia virus may be regarded as representative All recognizable virus particles disappear immediately after infection Some hours later the appearance of an electron-dense matrix area, contiguous with the nucleus, is the first indication of virus activity Virus particles subsequently emerge from this matrix, passing from developmental to mature forms which are ideal for electron microscope study due to their large size of 210 to 260 mμ and their characteristic oval shape surrounded by a double membrane The particles emerge in groups at the expense of the matrix area A peculiar feature of vaccinia virus is that it induces dense inclusion bodies within the cornea and more diffuse matrix areas within cells of the chorioallantoic membrane This general situation may be true of all poxviruses Thus, fibroma virus forms discrete inclusions in cells of cottontail testes (Kilham, 1956), but I have not encountered similar inclusions in rabbit kidney cells although the virus grows well in these latter cultures It is difficult to understand on what factors such differences are based Gaylord and Melnick (1953) simply state that a perinuclear area of viroplasm is formed in all cases but, depending on little understood host-parasite relations, discrete, dense inclusion bodies form in certain types of epithelium while more diffuse areas of viroplasm form in other types of cells

Febvre *et al* (1957) have described the intracellular development of fibroma virus with some precision They were able to infect rabbit testis cells at a definite time using special techniques, and to discover that 30 minutes was sufficient for virus attachment and/or entry Areas of viroplasm appeared 12 hours later (Fig 2) A finding of interest was that no infective virus could be seen or recovered until after 8 hours, when elementary bodies began to emerge from the viroplasm Within 24 hours the matrix was thin and crowded with virus particles Virus particles, at a later stage within the inclusion body, are shown by Fig 3, a photograph taken by Dr Jack Dalton while at the Sloan-Kettering Institute

E Squirrel Virus (SQV)

Fibroma and myxoma viruses are natural parasites of cottontail rabbits (*Sylvilagus* sp) from North and South America, respectively, and a closely related agent, SQV, induces tumors of natural occurrence in American gray squirrels (*Sciurus carolinensis*) This relationship is evidenced by serological reactions and the histology of the tumors induced

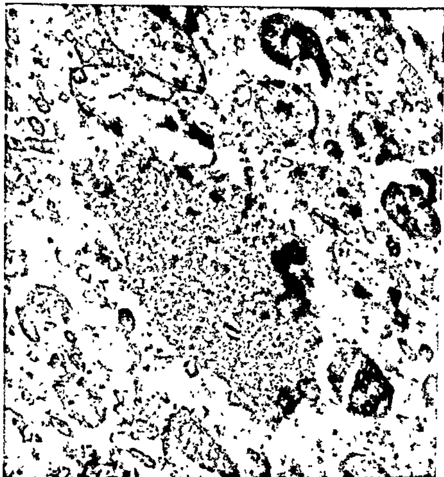


FIG 2 Area of viroplasm 12 hours after invasion of tissue culture cell by fibroma virus (×38,900) (from Febvre *et al*, 1957)

(Kilham *et al*, 1953) as well as by the type of insect transmission (Kilham, 1955) SQV differs principally in pathogenicity It does not grow, for example, in cottontails, it will grow for one passage only in domestic rabbits In squirrels it induces not only local skin tumors but leads to metastases widespread in skin and lung (Kilham, 1955) and the lung metastases bear some resemblance to human pulmonary adenomas (Kirchstein *et al*, 1958) A finding of interest has been that SQV is effective in transforming rabbit myxoma virus (Kilham, 1958) and its special characteristics offer a varying approach to problems of transformation

III. Experiments with HEAT-TAM

A. Transformations in Tissue Culture

Observable facts relating to transformation are simple in outline (Kilham, 1957, 1958) TAM or transforming agent myxoma is prepared from ground myxoma tumors by heating a suspension for 12 to 40 minutes at 65°C in a water bath. This HEAT-TAM is then added to cultures of rabbit kidney cells along with live fibroma virus Inactivated calf serum is also added prior to incubation at 36°C and harvests are made of supernatant fluids at 2 to 3 and at 5 days The entire cultures are frozen for harvesting at the end of a week Advanced CPE is usually present by this time In the course of a typical experiment, tissue culture fluids harvested at 2 to 3 days induce benign fibromas in test rabbits, whereas the 5- and 7-day harvests lead to myxomatosis HEAT-TAM is inoculated into parallel tissue cultures as a control It has never led to any definite CPE nor have fluids harvested from control cultures ever led to local or systemic signs of infection in test rabbits

A few earlier experiments were performed in explants of rabbit testis A finding of interest in these cultures, which clung to the glass for over 4 weeks, was that fibroma virus alone was recovered from fluids harvested during the first week and myxoma from four fluids harvested from the ninth to the sixteenth days Fibroma virus was again recovered over the next 16 days (Table I) Virus titers in rabbit skin reflected this transformation Thus titers of supernatant fluids averaged 10^{-2} when only fibroma virus was being recovered and 10^{-5} when myxoma was present (Kilham, 1957) One can only speculate as to why the more virulent agent appeared and then disappeared It seems probable, however, that transformation to myxoma may take place only in certain portions of the cell sheet and if these areas fall off due to the CPE of the myxoma and aging of the culture, only areas infected with the initial fibroma virus would remain

A question of importance has been whether transformation takes place in the tissue cultures or in the rabbits The increase in virus titers on appearance of myxoma, as described above, is one indication that it is initiated in the cultures Other evidence points in the same direction For example, once tissue culture fluids become positive for myxoma, succeeding fluids are also positive and no one, to our knowledge, has obtained such consistent results in rabbits alone Test rabbits must receive little of the original TAM after tissue cultures have had repeated changes of nutrient fluid

Experiments performed in rabbit kidney cultures have proved to be



Fig 3 Late stage of inclusion body showing developing form of myxoma virus (Electron photomicrograph ×60,000 by Dr J Dalton)

consistent and reproducible The transformation, however, does not depend on rabbit cells It has also been induced in cultures of monkey and of squirrel kidney, but results obtained with the former cultures were irregular in that only four of nine experiments were positive. A

TABLE I

RESULTS OF TESTS AND TITRATIONS ON SUPERNATANT FLUIDS TAKEN SERIALLY FROM TISSUE CULTURES OF TWO TRANSFORMATION EXPERIMENTS

Experiment	TAM added	Days after inoculation of tissue culture								
		4	7	9	11	14	16	18	25	32
BD 32	With fibroma virus	FIB[a] 10^{-2}[b]	FIB 10^{-3}	MYX 10^{-4}	MYX 10^{-4}	MYX 10^{-3}	MYX 10^{-3}	FIB 10^{-3}	FIB 10^{-1}	FIB 10^{-1}
35	4 Days after fibroma virus	FIB 10^{-1}	FIB 10^{-3}	FIB 10^{-3}	FIB 10^{-2}	FIB 10^{-3}	FIB 10^{-3}	FIB 10^{-3}	FIB 10^{-1}	FIB 10^{-1}

[a] Type of disease produced in test rabbit

[b] Virus infectivity titers of the fluids tested



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C Effect of Organic Solvents

TAM can be prepared by agents other than heat ETHER-TAM consists of myxoma virus exposed to anesthetic ether for 1/2 to 2 1/2 hours at room temperature and it contains no live virus demonstrable by rabbit or by tissue culture tests These TAM's have proved to be as active in transformation as the HEAT-TAM's A point of interest is that these ETHER-TAM's exhibit no diminution of activity when doubly "killed" by heating at 65°C for from 12 to 40 minutes The action of the ether is not known It may remove lipid as well as denaturing protein of the outer coat of the myxoma virus

Smith (1952) found that TAM could be prepared by extracting myxoma with chloroform Her statement, however, is made in only a few lines and without details of procedure or presentation of actual results, we have not been able to reduplicate her work In our experience myxoma virus suspensions treated with chloroform have lost both infectivity and transforming capacity This was shown in 10 tissue culture experiments Smith's results were apparently obtained in rabbits

D Effect of Enzymes, Light, and Other Factors

HEAT-TAM has proved to be a stable substance, resistant to prolonged heating and to ether as well as to the action of trypsin and of deoxyribonuclease (DNAase) It was of interest, therefore, to discover that it could be destroyed by light of various wavelengths Two HEAT-TAM's lost their transforming capacity when irradiated with intense visible light in the presence of toluidine blue O This reaction is presumed to be a photosensitized oxidation of the portions of the virus to which the dye is bound, namely, the nucleic acid Ultraviolet light has also destroyed HEAT-TAM This was under conditions which did not completely destroy the infectivity of suspensions of myxoma virus

E Ability of HEAT-TAM to Attach to Cells

HEAT-TAM is biologically inert in tests for infectivity and ability to immunize, but it is able to attach to and possibly enter tissue culture cells alone This could be a passive process The attachment has been demonstrated by means of wash experiments in which HEAT-TAM was allowed to overlay tissue cultures for varying periods in the presence of calf serum An effort was then made to wash it from the sheet of rabbit kidney cells by means of three changes of nutrient fluid HEAT-TAM could be removed in this way after 5 minutes It could not be washed

further finding has been that the Berry-Dedrick phenomenon does not depend on the Shope fibroma virus. Squirrel virus (SQV) has led to transformations with HEAT-TAM in cultures both of squirrel and of rabbit kidney (Kilham, 1958). Repeated attempts with vaccinia virus have been uniformly unsuccessful.

Hanafusa *et al.* (1959) have recently described the transformation of ectromelia into vaccinia virus in tissue culture. Their methods were comparable to those described above. Vaccinia virus, heated at 56°C for 3 hours was used as the transforming agent and the two viruses could be distinguished by the fact that vaccinia produces lesions in rabbit skin whereas ectromelia virus has no such effect.

B. Heat-Inactivation

TAM has been prepared by heat-inactivation of myxoma virus. This HEAT-TAM has proved to be a remarkably efficient transforming agent both in itself as well as in serving as the basis of UREA-TAM, a DNase-sensitive preparation discussed in a following section. Some of the properties of HEAT-TAM are discussed below. They have been reported previously by Kilham (1958) and Kilham *et al.* (1958).

Myxoma virus has been inactivated routinely by submergence in a water bath, held at 65°C., for from 12 to 40 minutes, although 3 minutes of exposure to this temperature are sufficient to "kill" the virus. Loss of infectivity has been demonstrated by two methods, (1) by direct inoculation of rabbits and (2) by passage of HEAT-TAM in tissue culture for 10 days prior to rabbit inoculation. Neither method has provided evidence that preparations contain infectious virus. An early question was whether the test rabbits might not have developed a subclinical or inapparent infection following intradermal inoculation with HEAT-TAM. Such an infection would, expectedly, immunize the animal to some degree. Rabbits challenged with live myxoma virus 9 to 12 days after previous inoculations with HEAT-TAM have, however, developed myxomatosis following a normal incubation period of 5 days. HEAT-TAM appears to be noninfectious as well as nonimmunogenic. A further question was whether large amounts of inactive virus particles *might not obscure the effect of a small fraction of surviving live particles.* This hypothesis was tested by inoculating rabbits with dilutions of HEAT-TAM, to a titer of 10^{8}. These animals likewise developed no signs of infection. An indication of the stability of HEAT-TAM is that preparations heated at 65°C. for 45 minutes have been just as active in transformation as aliquots heated for only 6 minutes.

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off after a 15-minute or longer exposure and its presence in the cells was indicated by the development of transformation following a subsequent addition of live fibroma virus This live virus could be added 24 hours after exposure of the cell sheet to HEAT-TAM These experiments suggest that the TAM particles do not depend on the fibroma virus for effecting fixation by the cells and also that they are relatively stable in the tissue culture environment

F The Nature of HEAT-TAM: A Reconstructed View

The essential structures of myxoma virus have been outlined by electron microscopy Farrant and Fenner (1953) found that the virus *particles had dimensions of 230 by 280 mμ* and that they *possessed an* outer membrane and a round, inner core of intense electron-scattering power Earlier studies by Dawson and McFarlane (1948) had revealed a similar structure for vaccinia virus The pepsin-resistant inner core encountered in both viruses probably consists of DNA. This was demonstrated in the electron microscope studies of Peters (1956), who found that it disappeared following exposure to papain and DNAase Additional evidence that poxviruses have a nucleus of DNA comes from other sources These include the original findings of Smadel and Hoagland (1942) as well as our studies on UREA-TAM (Shack and Kilham, 1959)

HEAT-TAM, in our view, represents myxoma virus particles with outer coats of denatured protein The TAM particles can be sedimented by the same centrifugation procedures as the infectious virus, they are not destroyed by DNAase, and they are relatively indestructible by such treatments as heat, ether, and exposure to a tissue culture environment On the other hand, HEAT-TAM is readily inactivated by photodynamic action. This procedure is considered to be specific for nucleic acids The aim of continuing research, discussed below, has been to remove the outer coats of denatured protein from particles of HEAT-TAM in order to expose, without destroying, the inner nucleus of DNA

IV DNAase-Sensitive TAM and Its Biological Properties

A Effect of Urea Treatments

UREA-TAM's have been prepared by dialyzing HEAT-TAM's against 10 volumes of 10 *M* urea in buffered saline for 8 hours at room temperature and then submitting them to further dialysis in buffered saline at 4°C (Shack and Kilham, 1959) The transforming ability of resultant preparations does not appear altered to any significant degree Thus, UREA-TAM has led to transformations in nearly 100% of experiments

when used in volumes of the same magnitude as those found effective for HEAT-TAM, namely, 0.1 to 0.4 ml. for each 2-ounce prescription bottle containing the tissue culture. Exact titrations of TAM activity have not been attempted. A primary difference between UREA-TAM and HEAT-TAM is revealed by their reactions to deoxyribonuclease, as shown in Table II. DNAase completely destroys the transforming ac-

TABLE II
Comparison of Effect of RNAase and DNAase on Transforming Activity of UREA-TAM

TAM lot numbers	Treatment	Enzyme used on TAM	Transformation experiment	
			Positive*	Negative
58, 72, 74	Heat† alone	DNAase	4	0
73, 74	Heat† + urea	DNAase	0	6
73, 74	Heat† + urea	RNAase	7	0

* Positive = transformation of fibroma to myxoma virus took place in tissue culture; negative = no change in tissue culture.
† 65°C., 12 minutes

tivity of UREA-TAM under conditions in which it had no effect on HEAT-TAM. This action appears to be specific for DNA. Aliquots of the same lots of UREA-TAM retained full activity after exposure to ribonuclease (RNAase). A continuing routine has been to assay all lots of UREA-TAM for sensitivity to DNAase; 47 of 48 experiments performed with 12 lots of UREA-TAM in adequate dosage were positive for transformation while 42 of 42 experiments carried out with aliquots of the same lots exposed to DNAase were negative.

One presumes that UREA-TAM represents particles of myxoma virus, of which the outer coats of protein have been denatured and then partially removed, thus permitting access to their inner nuclei of DNA. UREA-TAM can be sedimented in a Spinco in the same manner as HEAT-TAM. We have tried other methods for breaking down the particles of HEAT-TAM. One lot of TAM treated with sodium lauryl sulfate at 60°C. resulted in material which retained its transforming capacity while being sensitive to the destructive action of DNAase. Ten attempts to repeat this treatment were unsuccessful. It is possible that conditions involved were too critical for easy repetition. Phenol extractions of HEAT-TAM, as far as we have tried them, have completely destroyed transforming activity.

A consideration of importance in the above experiments was whether cultures of rabbit kidney cells might not produce DNAase spontaneously.

Investigations revealed that the cells do release DNAase I into the medium in amounts of 2.8–3.0 units per milliliter and that these amounts were fairly constant in fluids harvested every 2 to 3 days over a 2-week period. The findings have led to some modifications in procedures. We have washed cell sheets with fresh tissue culture fluid in an effort to eliminate some of this spontaneous DNAase activity prior to the addition of UREA-TAM.

UREA-TAM's have been tested for infectivity in the same manner as HEAT-TAM's. The original findings of Shack and Kilham (1959) have been extended so that 13 lots of UREA-TAM have been tested in cultures of rabbit kidney as well as by intracutaneous and intratesticular inoculations of rabbits. Inoculated rabbits have had no greater resistance to challenge with live myxoma virus than normal animals. It would appear that UREA-TAM, like HEAT-TAM, is both *noninfectious* and *nonimmunogenic*. A further attempt to recover live virus was made by inoculating UREA-TAM intracerebrally into mice, rats, and hamsters less than 24 hours old and, after harvesting a week later, inoculating the brains of these animals into rabbits. The animals all remained healthy and no myxoma virus was recovered. Myxoma virus is known to multiply in the brains of suckling mice (Andrewes and Harisjades, 1955).

B. Behavior In Different Types of Tissue Cultures

UREA-TAM differs from HEAT-TAM in biological properties other than destructibility by DNAase, as shown by its lability under different conditions of tissue culture. The transformation experiments described in this section represent unpublished data of Kilham and Shack.

HEAT-TAM is able to attach or enter rabbit kidney cells alone. This takes place within 10 to 15 minutes and, once fixed, HEAT-TAM remains undestroyed for as long as 3 days as indicated by the occurrence of transformation following the addition of fibroma virus. These results have been reviewed in a separate section above. When wash experiments were repeated using UREA-TAM, results obtained were less consistent, as illustrated by two sets of experiments presented in Table III. In a first set of experiments, UREA-TAM 76 was washed off after 10 minutes, but at 15 minutes it had become sufficiently attached so that it could not be removed. UREA-TAM 80, used in the second set of experiments, was washed off in periods up to 15 minutes. A feature common to both sets of experiments was that these UREA-TAMS, once attached to cells, could be identified for only a limited time thereafter. Thus UREA-TAM 76 was lost after the 10-minute period and UREA-TAM 80 after the 15-minute period, as shown by repeated experiments made to identify them at subsequent intervals. The situation was reversed in control

TABLE III

RESULTS OF EXPOSING UREA-TAM TO SHEETS OF RABBIT KIDNEY CELLS, THEN ATTEMPTING TO REMOVE IT AT DEFINITE INTERVALS OF TIME

Material exposed	Paired transformation experiments	Periods of exposure							
		5 min	10 min	15 min	30 min	2 hr	3 hr	4 hr	6 hr
UREA-TAM 76	Original	−[b]	−	+	−	ND	ND	ND	−
	Sub exp[a]	+	+	−	−	ND	ND	ND	−
UREA-TAM 80	Original	ND	−	−	ND	+	+	−	ND
	Sub exp	ND	+	+	ND	−	−	−	ND
Fibroma virus control	Original	−	+	ND	+	+	ND	ND	ND
	Sub exp	+	+	ND	+	+	ND	ND	ND

[a] Fluids poured from original bottle tested for TAM activity

[b] Negative (−) indicates no transformation took place
Positive (+) indicates transformation fibroma to myxoma virus

experiments in which fibroma virus was allowed to overlie the cells for varying periods of time. As shown in Table III, attachment or entrance of the virus alone took place within 15 minutes and it was not removable by washing after that period. One may conclude from these experiments that (1) UREA-TAM can attach to or enter cells alone but (2) it gets destroyed in this position within a fairly short time. Variation in results obtained was due, presumably, to the variability of the tissue cultures These were prepared weekly from fresh rabbit kidney, but, even, in regard to gross appearance they were never exactly the same from week to week.

The lability of UREA-TAM was further demonstrated in experiments with different types of tissue cultures, some of rabbit and others of nonrabbit origin. HEAT-TAM was effective in both types of cultures. As shown in Table IV, UREA-TAM led to transformation only in kidney cultures of lapin origin, whether they were derived from domestic or from cottontail rabbits, but was ineffective in cultures of squirrel kidney and of rat embryo. This differential in activity between rabbit and nonrabbit cells took place regardless of whether the Shope fibroma or SQV was used as the live virus in the transformation experiments Factors which may have played a role in these results have been the subject of continuing study. *They may involve a failure of UREA-TAM to* attach to or enter the cells of nonrabbit origin, a more rapid destruction after entry, or a combination of circumstances.

V. Perspectives

Investigations of the fibroma-myxoma transformation have, in one sense, only just started, but one can foresee progress in a number of directions which are all of theoretical interest. Two of these directions are indicated by the work of other investigators in related fields. A consideration of general principles involved may serve not only to give perspective on continuing work but also to point up the unique opportunities presented for research in the interactions of a live infectious virus, the DNA moiety of another, and mammalian host cells which can be stimulated into tumor formation under certain conditions.

A. Viral Recombination

The role of viral nucleic acids which has most attracted some investigators is its ability to relay genetic information from one generation to another. This function has not been clearly demonstrated for any animal virus It would appear, however, that the stage has already been set for such a demonstration by the work on virus recombination which has been going on for some years. The general principles involved

TABLE IV

NUMBER OF POSITIVE AND NEGATIVE TRANSFORMATION EXPERIMENTS IN TOTAL NUMBER PERFORMED WITH HEAT-TAM AND WITH UREA-TAM IN TISSUE CULTURES OF RABBIT AND OF NONRABBIT ORIGIN

Live virus	TAM preparation	Tissue culture (rabbit origin)				Tissue culture (nonrabbit origin)			
		Domestic rabbit (kidney)		Cottontail (kidney)		Squirrel (kidney)		Rat (embryo)	
		Number Positive	Number Negative	Number Positive	Number Negative	Number Positive	Number Negative	Number Positive	Number Negative
Shope fibroma	Heat	36	0	4	0	10	1	6	0
	Urea	33	1	4	0	1	10	0	7
Squirrel fibroma (SQV)	Heat	6	1	4	0	1	1	5	1
	Urea	12	1	4	0	0	4	0	6

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A classic example of infectious RNA is afforded by the work on the tobacco mosaic virus (TMV) As discussed by Fraenkel-Conrat (1959), TMV-RNA can be prepared so as to be over 99% pure, but this purified RNA is inefficient when compared with the whole virus as an infectious agent It takes 200 times as much of it to produce the same effect A surprising thing is that it is infective at all considering its sensitivity to enzymes, salts, traces of metal, and other environmental factors

An infectious RNA prepared from poliovirus was found by Holland *et al.* (1959) to have a wider range of infectivity than that of the intact agent The infectivity of poliovirus is limited to cells of primate origin The RNA derived from the virus, on the other hand, will infect a variety of cells of nonprimate origin, both *in vivo* and *in vitro* This latter production of whole virus particles is for one generation only Once the intact virus has reappeared, it is unable to infect the nonprimate cells which produced it, presumably due to limitations of action between the protein coat of the virus and surface receptors on the cell (McLaren *et al*, 1959)

If one thinks in comparative terms, UREA-TAM should represent the infectious DNA of myxoma virus, but it has never proved infectious either *in vivo* or *in vitro* One can only speculate on reasons for this lack of infectivity An initial consideration is that myxoma is among the largest of viruses, whereas infectious RNA's have come almost entirely from smaller viruses The nucleic acid of myxoma virus may be in the form of a loose structure of a number of molecules of DNA, and exposure to urea, possibly by its effect on associated protein, may disrupt this structure sufficiently to destroy its capacity for replicating new virus by itself This disruption, theoretically, only goes so far. It does not affect the capacity of the DNA to continue as an effective agent in transformation

A second consideration is that the exposed DNA of myxoma virus may be destructible by environmental factors such as enzymes, salts, and traces of metal, in the manner of TMV-RNA (Fraenkel-Conrat, 1959) This lability of UREA-TAM is, however, somewhat restricted in tissue culture. It can survive alone in cultures of rabbit kidney for from 1/4 to 3 hours Its sharpest difference in behavior from HEAT-TAM is that UREA-TAM has been fairly ineffective in certain cultures of non-rabbit origin Fibroma and myxoma are rabbit viruses The situation is, therefore, the reverse of that described for poliovirus which is limited to primate cells, whereas its infectious RNA will infect cells of non-primate origin (Holland *et al*, 1959) One interpretation of these phenomena is that myxoma virus has become adapted to survival in rabbit cells by the course of its previous evolutionary history It is

have been summarized by Burnet (1958). First, one needs two strains of virus which are closely related but differ in two or more well-defined and related markers, and second, combinants between the two strains must be tested for by passage in limiting dilutions in order to obtain them in pure clones. These methods have worked well with influenza viruses. All influenza A strains show potentialities of interaction and the investigations of Burnet (1958) and of Hirst and Gotlieb (1953) offer many examples These phenomena are also demonstrable for larger viruses. Gordon and Mamay (1957) have obtained combinant forms from two strains of psittacosis virus which differed in drug resistance. An advantage in such a system is that one can arrange a selective environment in embryonated eggs by using various combinations of the drugs involved Fenner and Comben (1958) have carried out genetic studies with mammalian poxviruses They have been able to demonstrate recombinant forms with different strains of vaccinia virus grown on the chorioallantoic membrane of the developing hen's egg, using such markers as pock morphology, heat resistance, and hemagglutination. Recombination was not an uncommon event in these experiments.

Genetic experiments on animal viruses have not depended entirely on living parent strains Baron and Jensen (1955), for example, obtained recombinants when using one strain of influenza A as a living infectious virus along with a differing strain which had been rendered noninfectious by exposure to ultraviolet light. Fenner and associates (1959) have used living and heat-killed poxviruses They were unable, however, to demonstrate recombination among the markers they were looking for and believed that the heat-"killed" viruses were simply reactivated by the living ones Their experiments, which were performed *in vitro* and *in vivo*, are of considerable interest to the problem of the fibroma-myxoma virus transformation, and one would be interested to see them continued with the use of partially purified DNA preparations. It is evident from Fenner's earlier experiments that strains of vaccinia virus are ideal subjects for studies in genetic recombination

B. Infectious Viral Nucleic Acids and UREA-TAM

Problems of more immediate interest in regard to the fibroma-myxoma virus transformation have not been genetic studies, for which no good markers are available, but in other activities of the viral DNA. The following are among questions which might guide continuing research: "Why is not UREA-TAM infectious by itself? What is the function of fibroma virus in the Berry-Dedrick phenomenon?" In seeking answers to these questions, it may be advantageous to consider a few of the facts relating to infectious nucleic acids in other viral systems.

et al (1959) have recently demonstrated the myxoma-immune as well as vaccinia-virus-immune rabbit serum react with other pox viruses in complement fixation tests This is additional evidence for the close relationship which exists among the Pox group of virus Fenner and Woodroofe (1960) have made the interesting suggestion that reactivation could be used as a taxonomic tool to identify new agents as belonging to the group, since a wide variety of unrelated viruses which they have used were without effect in induction of the phenomenon.

Woodroofe (1960) has found that the heat-inactivation of vaccinia virus does not always proceed in a regular manner. Thus the inactivation is a first-order reaction if the virus suspension is freshly prepared from the chorioallantoic membrane (CAM) or has been stored at —60°C Storage at 4°C leads to a heat-resistant form The explanation of this finding may lie with impurities associated with the virus, for the heat-resistant effect can be overcome by dilution

Joklik and co-workers (1960) have contributed information on reactivatable virus particles They used heat and exposure to urea as two different methods for inactivating rabbit-pox virus and showed that virus particles inactivated by either method might retain biological activity even after sizable parts of their outer portions had been digested away with proteolytic enzymes Electron microscope studies of these digested particles suggested that retention of a central body was essential to reactivation Additional findings of these workers appear to differ from those of Shack and Kilham (1959) Joklik and associates, for example, did not find that their urea-treated virus (U-RP) was sensitive to the destructive action of DNAase There are, however, three basic differences between U-RP and the UREA-TAM of Shack and Kilham (1959) First, the starting virus suspensions of the latter authors were cleared to a considerable degree by differential centrifugation prior to inactivation and second, they were heated at 65°C for 12 minutes before the urea treatment A third difference may have been of special importance As discussed in a separate section above, the DNAase sensitivity of our UREA-TAM was demonstrable only in tissue cultures of rabbit origin None of the U-RP preparations of Joklik and associates, on the other hand, were tested in cultures of this type One can summarize by saying that the Australian investigators obtained different results using different methods, and that much new light has been thrown on the Berry-Dedrick phenomenon in consequence of the variations in procedures used

Joklik *et al* (1960) have continued investigations along lines of considerable interest Their working hypothesis has been that the role of the live virus in reactivation experiments is to furnish an essential pro-

possible that the outer coat of the virus has some function in inducing the formation of the viroplasm, which serves as a protective zone, shielding the viral DNA from destructive forces within the cell. TAM of either type fails to replicate itself because it lacks an intact outer coat The fibroma virus with its intact outer coat is needed to form the viroplasm, which is essential to the replication of myxoma virus from TAM, but once the DNA's of the two viruses are together within one area, there may be opportunities for an exchange of genetic characters. One should not carry such speculations too far. It would appear, however, that performance of the Berry-Dedrick phenomenon in tissue culture offers varied opportunities for unraveling host cell-virus interactions.

VI More Recent Advances—An Addendum

Recent research, particularly from Australia and Japan, has given further understanding of the Berry-Dedrick phenomenon Hanafusa *et al.* (1959,b), for example, have found that the phenomenon is a general one among pox viruses and is demonstrable with vaccinia and ectromelia as well as with fowl pox, fibroma, and myxoma. In the case of the vaccinia-ectromelia system, the virus recovered has the same properties as the original vaccinia virus used as the heat-inactivated agent The studies of Hanafusa and associates confirm the findings of Kilham *et al.* (1958) in a number of details Among these are the observations that heat-killed virus can reach and persist in tissue culture cells by itself and that serum serves an essential function as a preserver of transforming potency when added to virus suspensions undergoing inactivation by heat. The Japanese workers have outlined conditions required for transformation in the following manner· (1) the active as well as the inactive virus should be able to grow in the cells used for the experiment, (2) the growth of the transformant is favored if it can be propagated more readily than the active form used along with it; and (3) *Hanafusa et al have made the interesting observation that transformation* does not necessarily proceed from a virus of less to one of greater virulence for, in the case of vaccinia and ectromelia, the reaction can move in either direction if one uses L or FL tissue culture cells in a selective manner. The general nature of the Berry-Dedrick phenomena has been demonstrated in considerable detail by Fenner and Woodroofe (1960). They have obtained transformation, or reactivation as they prefer to call it, with one-step mutants of some of the inactivated viruses, with other members of the vaccinia-variola group and by what they term the serologically unrelated pox viruses such as fibroma, myxoma, fowl pox, and contagious pustular dermatitis. One may wonder, however, whether these latter viruses are unrelated serologically. Takahashi

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tein component which is destroyed in the process of heating the inactive virus. This suggested that viruses inactivated by two different methods might be combined in one experiment. The viruses used included several strains of rabbit pox A suspension of one was inactivated by heat in the usual way and a suspension of another was inactivated by nitrogen mustard which is believed to destroy the inner DNA in a selective manner while sparing the outer protein coat. Reactivation took place between the two inactivated suspensions. The presumption was that the nitrogen mustard preparation supplied the necessary protein component while the heated suspension supplied an intact DNA Such results, among other things, suggest an approach to a study of the outer protein. It would appear from the variety of investigations which have stemmed from the original discoveries of Berry and Dedrick (1936a) that their phenomenon may provide a tool for investigating such fundamental problems as the following: the functions of outer viral protein; the methods of virus entry into cells; the possible ways in which viruses may multiply within cells, re-directing cell physiology to their own purposes, the interactions of viral proteins and inner DNA; and possibly, with new technique in the performance of experiments, the genetic mechanisms involved in recombinations among the pox group of viruses.

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THE BIOLOGY OF SOIL-BORNE PLANT VIRUSES

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I. Introduction

If, as many people consider, virology originated in Beijerinck's (1898) classic paper, in which he concluded that tobacco mosaic was caused by a type of infective agent quite different from bacteria, the study of soil-borne plant viruses is as old as virology itself Mayer (1886) had already suggested that the cause of tobacco mosaic should be looked for in the soil, but it was Beijerinck who first tested the suggestion and showed that tobacco seedlings contracted the disease when grown in soil collected some months previously from around the roots of a diseased plant A little later Behrens (1899) described the "Mauche" disease of tobacco (now often called tobacco rattle disease), concluded that it had a similar cause to tobacco mosaic and that infection depended on the soil, because he observed that infection occurred only in seedbeds on particular farms. For the next twenty-five years the idea that plant

TABLE I
A List of Soil-borne Plant Viruses

Virus	Species infected	Reference
Group A		
Tobacco mosaic	Tobacco	Beijerinck (1898)
Rotterdam-B	Tobacco	Thung and Hadiwidjaja (1958)
Group B		
Tobacco necrosis	Tobacco	Smith (1937b)
Cucumber necrosis	Cucumber	McKeen (1959)
Unnamed virus from *Chrysanthemum*	Tobacco	Brierley and Travis (1958)
Group C		
Wheat mosaic	Wheat	McKinney (1923)
Wheat yellow mosaic	Wheat	McKinney (1923)
Plains wheat mosaic	Wheat	McKinney (1953a)
Japanese wheat mosaic and wheat yellow mosaic	Wheat	Wada and Fukano (1937)
		Miyamoto (1958a)
Barley yellow mosaic	Barley	McKinney (1946)
Oat mosaic	Oat	Antoine (1957)
Sugarcane chlorotic streak	Sugarcane	
Group D		
Tobacco stunt	Tobacco	Uozumi (1954)
Group E		
Tobacco rattle	Tobacco	Boning (1931)
Group F		
Grapevine fanleaf		
European type	Grapevine	Petri (1929)
N American type	Grapevine	Hewitt (1956)
Grapevine yellow mosaic	Grapevine	Hewitt and Delp (1953)
Group G		
Tomato black ring (beet ringspot strain)	Sugar beet, etc	Harrison (1957)
Raspberry ringspot	Raspberry	Cadman (1956), Harrison (1956)
Arabis mosaic	Raspberry, sugar beet	Harrison (1958c)
Peach yellow bud mosaic	Peach	Wagnon and Breece (1955)
Cherry rosette	Cherry	Pfaeltzer (1959)
Others		
Peach rosette mosaic	Peach	Cation (1951)
Broad bean necrotic mosaic	Broad bean	Fujikawa (1955)

viruses could be soil-borne received little attention and it was not until 1931 that Böning established that tobacco rattle disease (which he called "Streifen und Kräuselkrankheit") was caused by a filterable, soil-borne virus.

About this time the pioneer studies of McKinney and Webb (McKinney, 1923, 1925; McKinney *et al.*, 1925; Webb, 1927, 1928) on wheat mosaic were re-establishing the idea that some plant viruses normally spread through the soil; this led to a detailed study of the role of soil-borne virus in outbreaks of tobacco mosaic disease (Johnson and Ogden, 1929). Work on grape *"arricciamento"* (a type of court-noué) showed that this disease too could be contracted from soil and suggested that its causal agent was a virus (Petri, 1929). These observations, and experiments on the mode of transmission of the tobacco necrosis viruses (Smith and Bald, 1935; Smith, 1937a,b), were almost the only major contributions to the study of soil-borne plant viruses up to 1945. Since then, however, the subject has developed rapidly as the number of recognized soil-borne viruses has increased and they have been shown to cause important diseases of a wide variety of crops.

Viruses that multiply in higher plants are not, of course, the only viruses found in soil The soil is one of the major sources of bacteriophages, which, like some animal viruses, for example, hog cholera (Shope, 1958), have hosts among soil-inhabiting organisms. There seems no unequivocal evidence that these viruses invade plants in nature, but some strains of the root nodule bacterium (*Rhizobium trifolii*) are lysogenic and such strains can infect legume root cells (Marshall, 1956). Furthermore, Murphy and Syverton (1958) reported that FA mouse encephalomyelitis and Type 1 poliomyelitis viruses were adsorbed by pea, tomato, and lettuce roots, and that FA virus occasionally was translocated to the plant shoots These viruses were not, however, shown to multiply in plants and they will not be discussed further

II. The Viruses as Plant Pathogens

For the purpose of this review "a soil-borne virus" is defined as "a virus with an underground natural method of spread which does not depend simply on contact between tissues of infected and healthy plants." Spread of virus by root grafting, which is thought occasionally to occur in trees (Hutchins, 1933), or by root contact, as appears to happen with potato virus X in potato crops (Roberts, 1948), are thus specifically excluded from further consideration

Table I lists the plant viruses found to be soil-borne, in the order in which they will be discussed below. The viruses within each group, although presumably not related strains, have many common attributes

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Sugarcane chlorotic streak	Sugarcane	Antoine (1957)
Group D		
Tobacco stunt	Tobacco	Uozumi (1954)
Group E		
Tobacco rattle	Tobacco	Böning (1931)
Group F		
Grapevine fanleaf		
European type	Grapevine	Petri (1929)
N American type	Grapevine	Hewitt (1956)
Grapevine yellow mosaic	Grapevine	Hewitt and Delp (1953)
Group G		
Tomato black ring (beet ringspot strain)	Sugar beet, etc	Harrison (1957)
Raspberry ringspot	Raspberry	Cadman (1956) Harrison (1956)
Arabis mosaic	Raspberry, sugar beet	Harrison (1958c)
Peach yellow bud mosaic	Peach	Wagnon and Breece (1955)
Cherry rosette	Cherry	Pfaeltzer (1959)
Others		
Peach rosette mosaic	Peach	Cation (1951)
Broad bean necrotic mosaic	Broad bean	Fujikawa (1955)

A. Tobacco Mosaic Virus Group

There is good evidence that tobacco mosaic virus in the soil can and often does play an important part in initiating outbreaks of mosaic in tobacco crops (e g , Johnson and Ogden, 1929), but it is not a factor in all such outbreaks. Tomato plants also can become infected with tobacco mosaic virus from the soil (Doolittle, 1928) but whether virus carried in the soil is of any importance in the ecology of other strains of tobacco mosaic virus, such as the cowpea strain which is found in wild plants in Nigeria (Lister and Thresh, 1955), the sann-hemp strain from India (Capoor, 1950), or the cucumber green mottle mosaic strain, is unknown The type strain infects tobacco seedlings whether it is added as infective sap or as infected roots to soil in which the seedlings are growing (McKinney, 1927)

Although Rotterdam-B virus does not appear to have antigens in common with the type strain of tobacco mosaic virus, both can cause similar necrotic diseases in tobacco and they are grouped together because they have particles of the same dimensions Rotterdam-B virus occurs in Sumatra, where tobacco is grown once in a seven-year rotation; the virus is soil-borne and it appears in the same areas each time a tobacco crop is grown (Jochems, 1928, Thung and Hadiwidjaja, 1958).

B. Tobacco Necrosis Virus Group

The first tobacco necrosis viruses to be described all caused similar symptoms in test plants, had similar *in vitro* properties, including thermal inactivation points in the range 70-95°C, and possessed isometric particles They were distinguished by the different kinds of crystals that formed in purified preparations and by their serological reactions (Bawden, 1941; Bawden and Pirie, 1942) For example, the Rothamsted culture had no antigens in common with the Cambridge culture, indicating that the different cultures were different viruses and not merely distinct strains of a single virus The viruses take their name from the appearance of the infected tobacco seedlings from which they were first isolated (Smith and Bald, 1935). In Britain, when tobacco seedlings are grown in unsterilized soil under glass in winter, necrotic symptoms often appear in the petioles and basal parts of the older leaves, whereas in summer the virus appears to remain in the roots and can only be detected there by inoculating indicator species In heated glasshouses, tobacco necrosis viruses also occur in the roots of many other species but do not invade the shoots The viruses are translocated to the tops of some plants (e g, of *Primula* spp) but remain localized and cause no symptoms. Tobacco necrosis viruses also cause a systemic necrotic

disease of tulip (Kassanis, 1949), known in the Netherlands as Augusta disease (de Bruyn Ouboter and van Slogteren, 1949) Few of the infected plants produce any saleable daughter bulbs, so that the disease is self-eliminating in tulip Its importance comes from the fact that symptoms seem often not to develop until the year after infection and thus infected, normal-looking bulbs can unwittingly be sold as healthy ones (Kassanis, 1954)

The Rothamsted culture is one of the best studied tobacco necrosis viruses, and, typically, it does not invade noninoculated parts of bean plants (*Phaseolus vulgaris*). However, a serologically related variant of this virus which can cause a systemic necrotic disease in runner (pole) bean occurs in the Netherlands (Bawden and van der Want, 1949), and similar diseases are known in Germany (Quantz, 1956) and the United States (Natti, 1959). There is also evidence that tobacco necrosis viruses occur in small amounts in many field soils (Cadman and Harrison, 1960), and that they cause small lesions on potato tubers, the "ABC disease" of Noordam (1957).

Although the tobacco necrosis viruses first described all caused very similar symptoms, symptomatologically distinct viruses with many affinities to tobacco necrosis viruses have recently been discovered One such virus was isolated in the United States by Brierley and Travis (1958) from *Chrysanthemum* and *Begonia;* it regularly causes systemic symptoms in tobacco plants and does not infect dwarf bean (*Phaseolus vulgaris*). Another, cucumber necrosis virus, causes a systemic necrotic disease of cucumber in Canada (McKeen, 1959) Cucumber necrosis virus is not serologically related to a Canadian tobacco necrosis virus that causes typical symptoms or to the *Chrysanthemum* virus (McKeen, 1959), but the viruses have many similar properties and are reasonably grouped together

C *Wheat Mosaic Virus Group*

This group of viruses with similar properties and ecology has been found only in cereals There is no critical evidence on the degree of relationship between viruses that have been differentiated by differences in host range and symptoms Wheat mosaic virus, which causes wheat rosette disease in certain wheat varieties (McKinney, 1923), Plains wheat mosaic virus (McKinney, 1953a; Sill, 1958), and wheat yellow mosaic virus have been described in the United States, where they are of great economic importance For example, in 1957 yields were decreased in value by about 4 million dollars in the state of Kansas alone (Sill and King, 1958) Wheat mosaic and wheat yellow mosaic occur in the same fields in the United States and diseases resembling them

have been described in Japan (Sawada, 1927; Wada and Fukano, 1934). Cells of infected plants contain characteristic inclusion bodies (McKinney *et al.*, 1923; Wada and Fukano, 1934). Oat mosaic virus has been recorded only in the United States (Atkinson, 1945; McKinney, 1946), and barley yellow mosaic virus only in Japan (Miyamoto, 1958a); neither infects wheat. Sugar cane chlorotic streak virus also may fall into this group, for there is evidence from Mauritius of infection from soil (Antoine, 1957) and it is restricted to Gramineae, but too little is yet known of its properties to be sure

All these diseases have proved difficult to study by usual techniques, and it could even be questioned that they are caused by viruses; there is, for instance, no evidence that juice from diseased plants is infective after passing through a bacteria-retaining filter. However, plants with wheat mosaic contain rod-shaped particles not found in healthy plants; the commonest length of these particles is about 130 mμ (Gold *et al.*, 1957).

D. Tobacco Stunt Virus

Tobacco stunt is a serious disease of tobacco in Japan but there is no unequivocal evidence that it is caused by a virus. The causal agent could not be transmitted by inoculation of sap and tobacco is its only known host In its apparently restricted host range and its behavior in soil, tobacco stunt virus resembles wheat mosaic virus. But unlike wheat mosaic virus, tobacco stunt virus is claimed to have small isometric particles (Hidaka *et al*, 1956)

E Tobacco Rattle Virus Group

Tobacco rattle virus, and the disease it causes in tobacco, have had many names since Behrens (1899) first called the disease "Mauche"; *Streifen und Krauselkrankheit* (stripe and curl), *ratel* (rattle), partridge, and others have been used, but tobacco rattle seems the most appropriate name for the virus because it was used when properties of the virus were first characterized (Quanjer, 1943).

Tobacco rattle virus has an extremely wide host range; it occurs naturally in many bulbous (van Slogteren, 1958) and nonbulbous (Uschdraweit and Valentin, 1956) ornamental plants, in several crop plants, and in many weed species (Noordam, 1956; Cadman and Harrison, 1959). An isolate from an *Atropa belladonna* plant in England, described as belladonna mosaic virus, is also a strain of tobacco rattle virus (Smith, 1943; Cadman and Harrison, 1959). Tobacco rattle virus apparently causes potato stem mottle, a disease that is often limited to one shoot on a plant (Rozendaal, 1947). Particular fields in the Neth-

erlands especially favor potato stem mottle or tobacco rattle diseases, and Rozendaal and van der Want (1948) found that potatoes contracted stem mottle when grown in a field where tobacco rattle disease had been prevalent Extracts from plants with each disease contained rod-shaped virus particles of similar dimensions, and inoculation of sap from potato plants with stem mottle caused rattle disease in tobacco (van der Want and Rozendaal, 1948) The specific particles are of two main lengths, about 70 mμ or 180 mμ (Paul and Bode, 1955), values which are particularly interesting because the larger of them is not a multiple of the smaller, and only the longer particles are infective (Harrison and Nixon, 1959). The particles are wider than those of tobacco mosaic virus but have a similar gross structure The two viruses also resemble one another in several of their properties, both are stable, readily purified, and have a high thermal inactivation point (Quanjer, 1943, Cadman and Harrison, 1959, Harrison and Nixon, 1959)

A feature of tobacco rattle virus not described with any other virus is that isolates differ greatly from one another in the concentration they achieve in inoculated tobacco leaves Some multiply so readily that a single local lesion yields enough virus to give hundreds of local lesions, whereas at the other extreme are isolates that multiply so poorly that they are difficult to maintain as single-lesion cultures Isolates with greatly differing abilities to multiply often occur together in one infected crop, but some soils seem predominantly infested with viruses that multiply readily and others with those that multiply poorly All the isolates will infect a range of hosts and all cause similar symptoms, but in none of these hosts do the indifferent multipliers reach a higher concentration than they do in tobacco The ability of the indifferent multipliers to multiply did not increase during 20 successive passages in tobacco. The isolates of ready multipliers, by contrast, always produce variant forms that multiply less readily than themselves, the poor multipliers derived in this way resemble those found in naturally infected plants and their ability to multiply does not change when they are passed several times through tobacco Cultures of ready multipliers differ in the frequency with which they produce variants that multiply poorly Some do so rarely and others so often that the variants may predominate in bulk cultures The *in vitro* properties of tobacco rattle virus have, perforce, to be determined with ready multipliers, because the others do not occur in amounts great enough for critical study However, sufficient has been done to suggest that the different cultures are variants of one virus (Cadman and Harrison, 1959).

It now seems clear that corky tissue in potato tubers is often associated with soil-borne viruses. This condition of the tubers was first

studied by Frank (1897) and Jensen (1900), and subsequently in many of the countries where potatoes are grown. Corky tissue in the tubers can take at least three forms: (a) small brown spots deep in the flesh of the tuber (these are a symptom of stem mottle disease); (b) arcs in the tuber tissue, often near the surface of the tuber (known as spraing, *kringerigheid, Pfropfenbildung*); and (c) large irregular patches deep in the tuber tissue (known as *Buntfleckigkeit*, sometimes as internal rust spot or *Eisenfleckigkeit*). These names have been used somewhat indiscriminately by different authors, making the literature on these symptoms as confused and confusing as it is extensive. The symptoms have been ascribed to many causes, ranging from lime deficiency, disturbance of respiration, and the use of fresh pig manure to bacteria and (as first suggested by Quanjer, 1926) viruses. Typically, few or no corky lesions develop in the tubers produced by plants themselves grown from corky tubers, behavior not immediately suggestive of a virus disease, but there is now considerable evidence to support Quanjer's suggestion that spraing symptoms [type (b) above] are caused by a virus. Both Atanasoff (1926) and Grieve (1934) claimed occasionally to have produced corky tissue in normal tubers by grafting them with cores from affected ones. Lihnell (1958) noted that affected plants occasionally produced one or more shoots with "spraing mosaic," a symptom somewhat like that of stem mottle disease. Tomato plants grafted with such shoots produced yellow line patterns on their leaves and when scions from these tomato plants were grafted on potato a few tubers developed corky, spraing-like symptoms. The symptoms in tomato are very like those caused by indifferently multiplying isolates of tobacco rattle virus, so attempts were made to detect such viruses by inoculating sap from corky tuber tissue to indicator plants, with some success (Cadman, 1959a; Cadman and Harrison, 1959). Also, Noordam (1956) noted that potato spraing occurred commonly in fields containing tobacco rattle virus and Eibner (1959) showed that tobacco rattle virus was present in all 31 of the spraing-inducing soils he tested but in only 1 out of 6 *Buntfleckigkeit*-inducing soils More important, Eibner induced superficial spraing symptoms by rubbing tubers with sap from tobacco with rattle. Thus earlier workers seem to have failed to isolate tobacco rattle virus from corky tuber tissue because the lesions contained too little virus; indifferently multiplying variants of this virus appear to be the commonest, and perhaps the only, cause of spraing Recent work also suggests that symptoms in potato cannot always be separated into typical stem mottle and typical spraing because intermediate forms also occur. There is as yet no evidence that internal rust spot [symptoms of type (c) above] is induced by viruses and it may well have physiological causes

Tobacco rattle virus is now recognized as being more important economically than previously supposed, for it causes common diseases in North America (Oswald and Bowman, 1958, Walkinshaw and Larson, 1958) as well as in many European countries.

F Soil-borne Grapevine Viruses

Vine growers in Europe, Africa, and America have long been troubled by abnormalities known in France as "court-noué" In addition to abnormally short internodes, the symptoms may include one or more of the following: two buds at a node instead of one, chlorotic markings on or malformation of the leaves, stunted growth, poor yield; and "endocellular cordons" Among the many causes of court-noué suggested by different authors are: adverse weather conditions, waterlogging of the soil; nutrient deficiencies or unbalance, copper toxicity; mites (Faes, 1905); endophytic mycorrhizal fungi (Rives, 1923); the fungus *Pumilus medullae* (Viala and Marsais, 1934); protozoa (Petri, 1923), and a virus or viruses (Petri, 1929) These conclusions were not necessarily all wrong, because the term court-noué has clearly been used in France to include almost any type of suboptimal growth; indeed by careful study of published accounts several syndromes can be distinguished Fanleaf and yellow mosaic are names given in the United States to two such diseases caused by viruses Fanleaf is very similar to and possibly identical with diseases called *arricciamento* in Italy, *Reisigkrankheit* in Germany, and *urticado* in Portugal: the European form of the disease was first shown to be contracted from soil by Petri (1929), a result subsequently confirmed by others, and it is transmitted by grafting (Johnsen, 1933, Branas, 1948). Vine yellow mosaic also is caused by a soil-borne virus (Hewitt and Delp, 1953) and seems very like the European diseases known as *"panachure," "mosaika,"* or *"clorosa infecciosa"* (Dias, 1955) Fanleaf and yellow mosaic viruses seem not to be closely related for Portuguese isolates do not protect plants from infection by one another in grafting tests (Dias, 1960), and still other viruses are also probably involved in the diseases covered by the general heading of infectious degeneration of the vine

Attempts to transmit viruses from grapevines by inoculation of sap have, with one exception (Ochs, 1958), failed, and the claims of Ochs to have isolated potato viruses X and Y, and cucumber mosaic virus from vines could not be confirmed (Niemeyer and Bode, 1959) Nevertheless, facts given below show that some grape viruses have affinities with the soil-borne ringspot viruses [1]

[1] North American grapevine fanleaf virus has now been transmitted by inoculation of sap from grapevines to a variety of herbaceous species Its *in vitro* proper-

G. Soil-borne Ringspot Viruses

The soil-borne viruses grouped under this heading have a wide host range, cause symptoms of the ringspot type in several of their hosts, can readily be transmitted by inoculation of sap, have similar *in vitro* properties (including thermal inactivation points of 50–70°C.) and at least three of them have polyhedral particles of about 30 mμ diameter (Harrison, 1958a, Harrison and Nixon, 1960).

At present this group includes tomato black ring, raspberry ringspot, arabis mosaic, cherry rosette, and peach yellow bud mosaic viruses. Strains of tomato black ring virus have been described under different names in different countries The type strain was isolated from tomatoes in England by Smith (1946), the potato bouquet strain from German potato crops (Kohler, 1952), and the beet ringspot strain from sugar beet, potato, turnip, oat, raspberry, strawberry, and weed plants in Scotland (Harrison, 1957, 1958b). Serological and plant protection tests showed that several Scottish isolates were closely related to one another, as were two English isolates, but the Scottish isolates were not closely related to English ones and still less so to a German isolate. The German isolate was closer to English than to Scottish ones. The degree of relationship between different isolates seems to reflect the degree to which their sources are geographically separated. Perhaps this behavior is to be expected more from soil-borne viruses, which have fewer opportunities than aerially transmitted viruses to become widely and rapidly dispersed, so that they will be more prone to geographical isolation and hence to evolve differently in different regions either in response to local conditions or by chance (Harrison, 1958b).

Raspberry ringspot virus, which causes "leaf curl," a disease that has devastated plantations of some raspberry varieties in Scotland (Cadman and Harris, 1952, Cadman, 1956), also occurs in variant strains in Scotland, England, and Germany and shows a similar type of geographical variation (Harrison, 1958a; Cadman, 1960). Strawberry is another important host, and raspberry ringspot virus is also at least partly responsible for some cherry diseases of the rasp leaf—Pfeffinger–Eckelrader type (Lister, 1958, Kunze, 1958, Cadman, 1960).

Other cherry rasp leaf diseases (Anonymous, 1959; Cadman, 1960) are associated with arabis mosaic virus, which was first isolated from a

ties resemble those of the soil-borne ringspot viruses and it shares some of its antigens with arabis mosaic virus Virus isolates from European grapevines, affected by diseases resembling fanleaf, have antigens in common with both North American fanleaf and arabis mosaic viruses (Cadman, Dias, and Harrison, *Nature* **187**, 577 (1960)).

plant of *Arabis* sp. growing in an insect-proof glasshouse at Cambridge, England (Smith and Markham, 1944). How this virus came to be in the Cambridge glasshouse has led to speculation about spontaneous generation of viruses, but there seems now no reason to suspect anything more unusual than that the virus was in the seed or the soil Arabis mosaic virus also causes serious diseases in raspberry and strawberry, its isolation from these plants led to it being called raspberry yellow dwarf virus (Harrison, 1958c), but later serological tests relate this isolate with the type strain of arabis mosaic virus (Cadman, 1960)

There is as yet no critical evidence whether cherry rosette virus, isolated from cherry with Eckelrader disease and shown to be soil-borne in the Netherlands (Pfaeltzer, 1959), is closely related to any of the other soil-borne ringspot viruses, but it causes ringspot symptoms in tobacco and other herbaceous species

Peach yellow bud mosaic virus is the only virus in this group yet found in North America, where it causes serious diseases of peach and almond in California (Thomas and Rawlins, 1951; Karle and Nyland, 1959). A year after being planted in infective soil, peach seedlings show symptoms (Wagnon and Breece, 1955) which somewhat resemble those caused by tomato black ring virus (Harrison, 1958d), but no serological relationship was detected between the two viruses[3] Peach yellow bud mosaic virus is readily transmitted from peach to cowpea and other herbaceous plants by inoculation of sap (Yarwood, 1956).

Two other soil-borne viruses, peach rosette mosaic virus (Cation, 1951) and broad bean necrotic mosaic virus (Fujikawa, 1955), are mentioned only in passing, for so little is known of their properties that their affinities with other soil-borne viruses, if any, are unknown By contrast with these two, there are several other viruses that appear from their general properties to have strong affinities with the soil-borne ringspot viruses, but whether they are soil-borne is unknown. It seems likely that the number of recognized soil-borne ringspot viruses will increase in the near future

Although more and more plant viruses are proving to be soil-borne, lettuce big vein, a disease contracted from soil and formerly thought to be caused by a virus, is now known to be associated with the chytrid fungus *Olpidium brassicae* (Grogan *et al*, 1958, Fry, 1958) How the fungus, which appears to remain in the roots, causes symptoms in the leaves is not clear, but a toxin, rather than a virus with unusual properties, seems likely to be involved

[3] Serological tests have shown that peach yellow bud mosaic virus is a strain of tomato ringspot virus (Cadman, unpublished observations)

III. Modes of Transmission

It is to be expected that the modes of transmission of soil-borne viruses will prove to be as varied and interesting as those of viruses that spread above ground, but at present this can only be postulated, for work on the subject is only now bringing its first results The transmission of no soil-borne virus has yet been elucidated in detail, but there is evidence that soil-inhabiting organisms transmit some. There is no such evidence with others, but with none can the soil fauna and flora be excluded as vectors, because infection from soil in bacteriologically sterile conditions seems never to have been claimed.

A Transmission without Evidence That Agents in Soil Are Needed

Tobacco mosaic virus is readily leached from infected tobacco tissues into soil, where it overwinters (Hoggan and Johnson, 1936); it can also overwinter in infected plant debris In the spring after a diseased crop the virus can be detected both in plant residues and in soil extracts (Johnson and Ogden, 1929), and field experiments indicate that overwintering virus is often a source of infection for the new crop. Although McKinney (1927) found that infection occurred when either sap or fresh roots from diseased plants were newly added to soil, the condition of the virus does greatly affect its ability to survive in soil for long periods, as also do soil conditions. In dried plant debris the virus was stable in dry, but not in moist, aerobic soil or sand· it was also stable in moist anaerobic sand When liquid extracts containing virus were added to dry soil, infectivity survived less well than when dried leaves were added. However, the infectivity of liquid extracts survived better in dry or waterlogged soil than in moist soil and it survived better in clay soils than in sand All these observations suggest that infectivity was lost most rapidly when the soil conditions most favored the activity of aerobic microorganisms, and that there may be a causal connection between the two Indeed, when bacteriologically sterile virus preparations were kept with moist soil in sterile conditions, little infectivity was lost in six months (Johnson and Ogden, 1929).

Plant viruses are adsorbed by, and can subsequently be eluted from, colloidal particles, notably clays, and this ability may affect their survival in soil (van der Want, 1952) Indeed, adsorption on clay may be a factor in explaining the longer survival of tobacco mosaic virus in clay soils than in sand (Johnson and Ogden, 1929). Different viruses are adsorbed by clay to different extents and Rotterdam-B virus (which may be a distantly related form of tobacco mosaic virus) is adsorbed more strongly than the type strain of tobacco mosaic virus (Thung

and Hadiwidjaja, 1958). However, further work clearly is needed before the importance of colloid-adsorbed virus can be assessed.

There is little evidence to show what fraction of the virus that survives in soil is at sites from which it is potentially able to infect plants or on how infection occurs. Primary infections presumably occur through wounds produced when tobacco roots scrape against soil particles; later, when it has multiplied and invaded the tops of a few plants, the virus readily spreads above ground to other plants. But tobacco mosaic virus seems not to move easily from root to shoot of tobacco, and in artificially inoculated plants the virus often remains in the roots for long periods (Fulton, 1941) The virus presumably behaves similarly in naturally infected plants, and Johnson (1937) concluded that in tobacco crops most primary mosaic infections of tops originate by contact of stems and leaves with the soil and that only a few occur through the roots

Although the great stability of tobacco mosaic virus and the behavior of the virus in laboratory experiments make it probable that plants in the field become infected from soil by a process of mechanical inoculation, the details of the process have still to be discovered

Even less is known about the transmission in soil of tobacco rattle virus than about that of tobacco mosaic virus Eibner (1959) showed that plants of tobacco, potato, and the weed *Stellaria media* became infected when growing in sterilized soil to which sap containing tobacco rattle virus was added However, in these conditions only a small percentage of potato tubers developed spraing symptoms, so it is questionable whether this manner of infection resembles that in naturally infested soils. Such artificially infested soil lost its ability to infect tobacco within a few weeks although kept in conditions in which naturally infested soils retained their infectivity for longer periods Also, as a few weeks of air-drying abolishes the infectivity of soil containing tobacco rattle virus (Eibner, 1959) but is less effective with soil containing tobacco mosaic virus, a purely mechanical explanation of the survival and transmission of tobacco rattle virus in soil may not be adequate[3] Tobacco rattle virus seems never to have been detected in extracts of naturally infested soils, but the fact that it is strongly adsorbed by clays (van der Want, 1952) may hinder its detection and greatly affect its behavior in soil.

Tobacco necrosis viruses have been mainly reported in glasshouse-

[3] Results recently obtained by R Gessner (Doctoral Dissertation, 1959, Justus-Liebig-Universität, Giessen, Germany) support the idea that soil-inhabiting organisms are concerned in the transmission of tobacco rattle virus For example, treatment with Vapam or heating for one hour at 50°C abolished the infectivity of soil but had little or no effect on the virus *in vitro*

grown plants, but they occur in the open and were present in most of the field soils tested by Cadman and Harrison (1960). Most infections pass undetected because the viruses are restricted to the roots, and the foliage of infected plants looks normal In glasshouses at Rothamsted, for some obscure reason infection of roots is always more prevalent among plants grown in unsterilized soil in winter than in summer. Smith (1937b) found tobacco necrosis virus in glasshouse water tanks, suggesting that pots were inoculated with the virus every time they were watered Indeed, this observation could explain the frequency of infection among Smith's control plants grown in previously autoclaved soil and kept in the glasshouse, a result not confirmed at Rothamsted Frazier (1955) concluded from work with *Fragaria vesca* that infection occurred when roots grew in contact with the moist surface of the glasshouse bench Thus the increased prevalence of the virus in winter may result partly from the fact that the glasshouse bench is then usually wet or moist for long periods and partly because the plants may be more susceptible to infection in winter.

Tobacco necrosis virus can often be detected in extracts of infested soil, but how much of this apparently free virus comes from root fragments is unknown; the virus remains infective for many weeks after infective sap is added to soil (Smith, 1937b) and plants become infected when grown in soil to which virus-containing sap or roots have been added Cucumber necrosis virus, too, infects cucumber plants when added to soil in infective sap or diseased roots (McKeen, 1959). Infection does not occur when plants are grown on filter paper moistened with suspensions of tobacco necrosis virus, suggesting that the virus may normally invade roots through wounds made during their growth through soil (Smith, 1937b). Perhaps the viruses can also enter roots through cells that have been damaged by soil-inhabiting organisms, but tests with the fungi *Rhizoctonia solani* and *Thielaviopsis basicola* gave no support for this idea (Bawden and Kassanis, 1947) Thus, although tobacco mosaic and tobacco necrosis viruses have such very different properties *in vitro*, their behavior in soils has many apparent similarities

The last virus to be included in this section, sugarcane chlorotic streak virus, seems to spread readily from plant to plant even when precautions are taken to prevent contact between roots This was shown by infection occurring in initially healthy plants when nutrient solution was circulated round a closed system of water-culture pots, of which alternate pots at first contained healthy and diseased plants (Bird *et al.*, 1958). Few comparable experiments have been made with other viruses, but Roberts (1950) found that potato virus X only spread between plants in culture solutions when their roots were in contact.

B Transmission with Evidence That Agents in Soil Are Needed

Although wheat mosaic is the best studied of the soil-borne cereal viruses, its mode of transmission has defied elucidation for many years The virus is unstable in plant sap, which loses infectivity in a few days at room temperature or when heated for 10 minutes at 65°C Plants remain healthy when grown in soil inoculated with infective sap, or with leaves or roots of artificially infected plants, but McKinney *et al* (1957) have made the interesting discovery that wheat became infected when grown in soil infested with *roots of naturally infected* wheat plants and not when only leaves were added Comparable experiments with oat mosaic virus gave the same results, from which it can be argued that something present in naturally infested soil and closely associated with the roots of naturally infected plants plays a part in virus transmission This something, which could be animal, vegetable, or mineral, evidently survives several years of desiccation, because dried soil remains infective for these periods (McKinney, 1923) It must also be of a size that will pass through a 250-mesh sieve (McKinney, 1953b) but not through cheesecloth or filter paper (Webb, 1928)

Other observations on the behavior of infested soil also are compatible with McKinney and associates' (1957) suggestion that the virus is transmitted by an organism, but they do not prove it Thus the critical evidence from American work on the possible existence of a vector comes only from the experiment in which soil became infective when roots from naturally infected plants were added to it, moreover, several workers have searched unsuccessfully for the agent among root parasites

The results in America are difficult to reconcile with those of Miyamoto (1958a,b, 1959a) in Japan, who studied the transmission of barley yellow mosaic virus, which has similar properties and seems to behave in soil in many respects like wheat mosaic virus, although it does not infect wheat When soil containing barley yellow mosaic virus was fractionated by the rate at which particles sedimented in water, the most slowly settling fraction, containing clay and organic matter, was the most infective for seedlings grown in it and, in contrast to wheat mosaic virus, barley yellow mosaic virus was detected by inoculating the leaves of plants with suspensions of this slowly settling fraction Whether the virus detected in this way came from tissue fragments or was free in the soil is not clear, but Miyamoto (1959b) showed that seedlings become infected when grown in kaolinite clay preparations to which infective sap had been added 8 months previously, although the mixture had been stored in conditions in which the infectivity of sap is lost in 3 days or less Miyamoto's observations show that barley yellow mosaic virus can be

detected in soil extracts, that when added to some types of soil particles in sap the virus remains infective for long periods and can infect plants, and that there is no need to invoke the presence of a soil-inhabiting organism to explain transmission. Indeed its similarity to wheat mosaic virus is the only good reason for discussing barley yellow mosaic virus in this section.

The causal agent of tobacco stunt appears to behave very similarly to wheat mosaic virus in soils Their respective host plants have a similar optimum temperature for infection and symptom appearance, and tobacco-stunt-inducing soils remain infective after 4 years of dry storage or after being passed through a 300-mesh sieve (Hidaka *et al.*, 1956). Here, too, the mechanism of transmission is uncertain.

There is now good evidence that an agent in soil is involved in the transmission of several of the soil-borne ringspot viruses. With tomato black ring virus the evidence is of three types. First, seedlings become infected when grown in naturally infective soils, although the virus cannot be detected in extracts from such soils, and attempts to make sterilized soil infective by adding large quantities of virus-containing sap or by adding roots from artificially infected plants failed Second, the infectivity of soils was abolished by chemicals (e g, tetramethylthiuramdisulfide) which appear not to affect infectivity of the virus *in vitro*. Third, some unsterilized virus-free field soils, but not others, became infective when cropped with artificially infected plants (although not when watered with infective sap); these soils were not receptive to arabis mosaic virus and their receptivity to tomato black ring virus was abolished by autoclaving The first sort of evidence has also been obtained with raspberry ringspot virus, which is probably transmitted in a similar manner to tomato black ring virus because the two viruses often occur in the same fields and in the same patches within these fields (Harrison, 1958a; Cadman and Harrison, 1960).

The agent in soil suggested by these experiments has, however, quite different properties from that apparently associated with wheat mosaic virus. Indeed, the infectivity of soils containing tomato black ring virus is destroyed by one week of air-drying at room temperature. Also, adding to soil roots of plants *naturally* infected with tomato black ring virus did not render it infective Thus the soil agent does not seem closely associated with roots, it is locally distributed in soils, and either it or the virus is inactivated by drying the soil Many types of soil organisms have the characteristics ascribed to this agent, among them migratory soil nematodes. Indeed, seedlings became infected with tomato black ring virus when watered with crude suspensions of nematodes from infective soil. These suspensions also contain other types of or-

ganisms and it is not yet certain whether or not the agent is a nematode (Cadman and Harrison, 1960), although this is plausible because nematode vectors for two similar viruses, peach yellow bud mosaic and arabis mosaic viruses, have recently been claimed (Breece and Hart, 1959; Jha and Posnette, 1959; Harrison and Cadman, 1959). Also, the infectivity of soils containing arabis mosaic virus behaves like that of soils containing tomato black ring virus in being abolished by air-drying and by tetramethylthiuramdisulfide (Harrison and Cadman, 1959)

Grapevine fanleaf was the first virus shown to be transmitted by a nematode (Hewitt *et al.*, 1958). The vector is a large migratory dagger nematode (*Xiphinema index* Thorne and Allen) with a long mouth spear. The first indication that dagger nematodes transmitted fanleaf virus was obtained by growing healthy and diseased plants in the same pots and adding *X index* to some of the pots, transmission occurred only when *X index* was present. The virus was also transmitted when hand-picked *X index*, collected from around the roots of diseased plants, were transferred to pots containing healthy plants, some transmission occurred with as few as five nematodes per pot but never in pots without *X index* or in pots infested with *X. index* from healthy fig roots. Both larvae and adult female *X index* can transmit (males of this species are rare) and some of the nematodes are still infective after periods of up to 30 days without access to plants The virus evidently persists in the nematodes, but there is no evidence that it is transmitted through the egg to progeny of infective mothers (Hewitt *et al*, 1958, Raski and Hewitt, 1959)

Other dagger nematodes have now been identified as vectors· one, *Xiphinema diversicaudatum* Micoletzky, transmitted arabis mosaic virus to strawberry (Jha and Posnette, 1959) and to pea (Harrison and Cadman, 1959), and another, probably *X americanum* Cobb, transmitted peach yellow bud mosaic virus to peach (Breece and Hart, 1959), when individuals hand-picked from soil around the roots of diseased plants were transferred to pots containing virus-free soil and healthy plants *Xiphinema* seems to be the major and probably the only vector of arabis mosaic virus in the soils studied, for when infested soil was fractionated by washing it through sieves with successively smaller mesh sizes, the material that passed through the sieve that retained the smallest *Xiphinema* larvae was not infective, and when *Xiphinema* were hand-picked from infective soil extracts, the residue was not infective Adult male, adult female, and larval *Xiphinema* all transmitted the virus, but whether adult as well as larval forms can acquire the virus is unknown (Harrison and Cadman, 1959)

It is too soon after the discovery of these eelworm vectors for the

mechanism of virus transmission to have been elucidated, but it seems clear that ability to transmit a given virus is probably specific to one or a few species and is not a property of any eelworm that feeds on plants (Schindler, 1958) The first three viruses found to be transmitted by eelworms all have species of *Xiphinema* as vectors, but several other eelworms seem equally well-fitted to be vectors and it is to be expected that other soil-borne viruses, as yet without known vectors, will be transmitted by other genera.

IV. Virus Ecology

A Interactions of Viruses and Plants

The tobacco necrosis viruses, tobacco rattle virus, and the soil-borne ringspot viruses all have an extremely wide host range, both natural and experimental It is rare to find a species immune to tobacco necrosis viruses, and tobacco rattle virus has been transmitted experimentally to over 400 plant species (Schmelzer, 1957) and isolated from naturally infected plants of more than 100 species (e g , Uschdraweit and Valentin, 1956, Noordam, 1956) Tobacco mosaic virus also has a wide host range and in tomato fields has been found infecting wild plants, e g , *Physalis* sp. and *Solanum* sp (Gardner and Kendrick, 1923). Thus it is not surprising that several of these viruses have been independently described from different host species and given a multiplicity of names Some soil-borne viruses are, perhaps, more plausibly considered as usually being pathogens of wild plants than of crops they happen to infect when these are grown on infested land By contrast, the cereal mosaic viruses have been transmitted only to species in the Gramineae, the grapevine viruses only to species in the Vitaceae and tobacco stunt virus only to tobacco Soils containing wheat mosaic or tobacco stunt viruses remain infective for several years without host plants and there is no reason to suspect that a wide host range is needed to ensure the survival of such viruses However, failure to find other hosts of the grape viruses may come merely from the difficulty of transmitting viruses from species that have acid sap (Cadman, 1959b, see footnote 1)

Restriction of infection to the roots is common with soil-borne viruses, particularly tobacco rattle and the tobacco necrosis viruses. Indeed, Yarwood (1959) has claimed that *Cleome* plants grow taller when their roots are infected with a tobacco necrosis virus than when they are not. But even the tobacco necrosis viruses do occasionally invade shoots, when they may (Smith and Bald, 1935) or may not (Bawden and Kassanis, 1947) cause symptoms. Restriction to the roots does not happen

because the shoots are immune and there is no evidence that soil-borne viruses cause the type of host reaction shown by the Saco variety of potato to potato virus S, where the virus, even when introduced into shoots by grafting with infected scions, accumulates only in the roots (Larson and Oshima, 1959) However, ability to multiply in shoots, but not in roots, is one possible explanation for the behavior of wheat varieties which are unaffected by wheat mosaic in the field although they show obvious symptoms when infected by inoculation of sap (McKinney, 1948)

The soil-borne ringspot viruses also are often confined to the roots of infected plants, but this is usually only when the plants have been recently infected and the virus has not yet had time to invade the shoots Raspberry ringspot symptoms rarely appear in the year that the roots of raspberry plants become infected, and only a small proportion of the tubers produced by a potato plant newly infected with tomato black ring virus are themselves infected, showing that the virus does not become generally distributed in the underground parts of the potato plant in the first year of infection (Harrison, 1959)

Few symptoms in the underground parts of plants, other than stunting, are attributable to soil-borne viruses The corky arcs found in potato tubers affected by spraing, the necrotic spots in hyacinth bulbs infected with tobacco rattle virus and in tulip bulbs infected by tobacco necrosis viruses are, however, notable exceptions (de Bruyn Ouboter and van Slogteren, 1949, van Slogteren, 1958) The roots of grapevine plants affected by diseases of the fanleaf type often carry small galls, which were once thought to be a symptom of infection (Petri, 1931) but are now known to be a response to the feeding of the nematode vector of the virus, *Xiphinema index* (Raski and Radewald, 1958) Many symptoms are produced by soil-borne viruses in plant shoots and, although none of these types is sufficiently characteristic to distinguish soil-borne from other viruses, some symptoms, such as chlorotic or necrotic ringspots, are caused by several soil-borne viruses.

Little is yet known about how environmental factors affect the susceptibility of plants to infection from the soil, but age of the plants is certainly important Webb (1927) transplanted seedlings from soil infested with wheat mosaic virus to virus-free soil at intervals after sowing, he also transplanted seedlings from virus-free to infested soil at intervals, and deduced from the rates of infection in the two kinds of experiments that seedlings were most susceptible when 1–4 weeks old Similar experiments showed that turnip seedlings were most susceptible to infection with tomato black ring virus during the first week after germination (Cadman and Harrison, 1960)

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B Behavior of Viruses in Soils

Several features of the behavior of viruses in soils have already been described in Section III (Modes of Transmission), but there are others that merit discussion The first is the relation between disease outbreaks and soil type Some viruses seem closely associated with a given type of soil: for example, tobacco rattle and tomato black ring viruses have been found only on light loams or light peaty soils (Rozendaal, 1947; Harrison, 1957), and wheat mosaic and sugarcane chlorotic streak viruses occur mainly on heavy soils (McKinney, 1925; Antoine, 1958). By contrast, arabis mosaic virus occurs on a wide range of soils including light shingle, medium-heavy clay, and black peaty fen (Lister, 1960a; Harrison and Cadman, 1959); infested shingle soil had a gleyed mixture of sand and clay at about 18 inches depth, suggesting that an important common factor between these soils may be resistance to drying out, because drying quickly kills the nematode vector.

The restriction of wheat mosaic virus to heavier soils may also indicate the importance for infection of a high soil moisture content. Webb (1927), using a fine sedimentary soil with a moisture-holding capacity of 60%, found that most infection occurred when the soil was kept at 52% of the moisture-holding capacity, a little at 42%, and none at 30%. Temperature was also important, most infections occurring at 16°C, fewer at 10°C, and none at 23° or 30°C

Soil-borne viruses are often patchily distributed in crops but their distribution in soil within the patches has been little studied. Eibner (1959) sampled adjacent areas (each 20-cm square) of a soil infested with tobacco rattle virus and found that infectivity tended to be concentrated in small patches of 40–60 cm. diameter. The top 10 cm or 20 cm of soil was the most infective and that below 40 cm was noninfective, showing that, in these cultivated podsols, maximum infectivity occurred only in the organic A-horizon Wheat mosaic virus was in the top 5 cm of soil, and at a depth of 60 cm in one soil, although not below 45 cm in another (Koehler *et al*, 1952, Sill, 1958). By contrast, the top 10 cm. of soil from around the roots of peach trees infected with yellow bud mosaic virus was not infective, whereas that from 10–45 cm. was (Wagnon and Traylor, 1957), possibly because the vector (*Xiphinema americanum*) was not common near the surface.

Even with virus-containing soils that readily lose infectivity, for example, those containing tomato black ring virus, soil samples collected at all times of the year were infective. Decreases in infectivity seemed related to long exposures to freezing temperatures, and pot experiments confirmed that infectivity was decreased by freezing (Harrison, 1958a;

Cadman and Harrison, 1960). Freezing also inactivates tobacco mosaic virus in soil, but not in undecayed plant tissue (Hoggan and Johnson, 1936). Viruses differ greatly in the extent to which they are affected by variations in soil conditions, presumably because their modes of transmission are radically different. Among the better studied viruses, tobacco rattle probably behaves least predictably in soils and wheat mosaic most predictably, infectivity being maintained at a high level for long periods.

C. Behavior of Disease Outbreaks

Outbreaks of diseases caused by soil-borne viruses are of two types The first is caused by virus established in the soil, and diseased plants occur in patches distributed in the crop; this type is most obviously shown in perennial crops, such as raspberry or grapevine, but also in wheat crops affected by mosaic When outbreaks derive from infested soil, weeds as well as crop plants usually are infected The second type of outbreak is caused by virus imported in infected planting stock; obviously this occurs mainly with vegetatively propagated crops, usually resulting in a roughly random distribution of infected plants in the crop Outbreaks of tobacco mosaic virus are, however, an exception to this generalization, because plants infected from the soil and showing mosaic symptoms are sporadically distributed, perhaps partly because the virus only invades the shoots of a few of the plants of which the roots are infected

There are few measurements on the rate at which soil-borne viruses spread through fields Observations on patches of diseased perennial crops give some information but the delay between infection and symptom development, which may be as much as two years in raspberry and perhaps longer in trees, complicates interpretations When many plants become diseased during the three or four years after planting perennial crops, delay in infection from already infested soil or delay in symptom expression is probably the reason rather than that the area of infested soil has greatly increased Spread of virus in older plantations is slow: for exam[illegible]
than tw[illegible]
raspberr[illegible]
rate, but some patches of diseased plants do not increase in size at all, although the soil within the patches remains infective (Cadman and Harris, 1952; Cadman, 1956) Spread of arabis mosaic virus in raspberry, strawberry, and clover crops seems limited by the distribution of dagger nematodes, which occurred only in patches where the plants were already infected (Harrison and Cadman, 1959). To what extent

rates of spread represent the independent movement of virus or virus vectors through the soil, and to what extent the movement of soil during cultural operations or of virus through plants, is unknown. Indeed, when a virus invades the root system of a tree, it will probably be distributed through many square yards of land within a few years. Spread may perhaps also result from the run-off of rainwater (McKinney, 1923).

The most obvious way in which soil-borne viruses seem likely to become established in the soil at new localities is by transfer of infective soil on agricultural implements, boots, etc ; Koehler *et al*, (1952) showed that attacks of wheat mosaic could become serious within four years of adding 1 part of infective soil to 10,000 parts of virus-free soil. As soils containing wheat mosaic virus do not lose infectivity when dried, dust storms could spread this virus (McKinney *et al.*, 1925; Koehler *et al.*, 1952) Smith (1937c) claimed that tobacco necrosis viruses are air-borne, for he obtained infections by rubbing leaves with moist cotton wool pads through which large amounts of glasshouse air had been drawn and on which dust and small fragments of plant debris presumably had been deposited He (1937b) also concluded that tobacco necrosis virus, which he detected by inoculating plants with sludge from the bottom of the glasshouse water tanks, could be spread in water used in the glasshouse Tobacco mosaic virus is spread far more readily by contact with contaminated hands or tools than through soil, so that its introduction to fields where the virus is not already in the soil is easily explained

An obvious way of soil becoming infested is by planting infected propagating material in it, but this depends on the "receptivity" of the soil For example, in glasshouse conditions tomato black ring virus did not become established when artificially infected plants were grown in previously sterilized soil, but it did in some unsterilized field soils. Field experiments extended these results by showing that when a systemically infected potato stock was grown in one such field, the virus became established in the soil and infected the subsequent crop of sugar beet (Cadman and Harrison, 1960) The receptivity of this soil presumably means that it initially contained the agent responsible for transmitting the virus. This method of spread to new localities may be common in agriculture and horticulture, for vegetatively propagated stocks of crop plants are frequently distributed over great distances Another method of long-distance spread has recently been discovered by Lister (1960b), who finds that tomato black ring virus is transmitted through the seeds of some hosts, including weeds. Some of these species, such as groundsel (*Senecio vulgaris*), have wind-dispersed seeds that may be carried over

great distances, but the persistence of the virus where the seed germinates will depend on the receptivity of the soil

Cultural practices undoubtedly affect the epidemiology of soil-borne viruses. Thus disk-cultivating the residues of tobacco crops in autumn resulted in their quicker decay and in more virus inactivation, and hence in less mosaic infection of the next crop from the soil, than disking in spring or deep ploughing in autumn (Lehman, 1934). Neither summer fallowing nor the use of different fertilizers seem to affect the incidence of wheat mosaic virus (McKinney, 1923), but fallowing may have a greater effect on the incidence of viruses that have nematode vectors However, the sequence of crops grown seems more important for the survival of wheat mosaic virus than, for instance, raspberry ringspot virus. Infestations of wheat mosaic virus built up to a high level when four successive wheat crops were grown, but decreased from a high to a low level when four successive oat, soybean, or maize crops were taken (Koehler *et al*, 1952) Raspberry ringspot, by contrast, occurred on patches of infested land when raspberry had not been grown during the previous ten years (Cadman, 1956).

V Control of Soil-borne Viruses

A thorough knowledge of the ecology of a disease and of its causal agent ideally should lead to the formulation of effective control measures But only a small part of this information is available for soil-borne viruses and many of the control measures used are empirical These measures comprise methods of preventing the introduction of viruses to new sites and of combating the viruses where they are already established

A Prevention of Spread to New Sites

Different soil-borne viruses, as already stated, probably spread to new sites in different ways, but whether the ways now known are the only important ones and whether precautions against them will be effective is uncertain. It is obviously desirable not to carry virus-infested soil to virus-free fields on farm implements, etc, and not to introduce tobacco mosaic virus on contaminated hands, clothes, and tools It is also desirable to ensure, when planting vegetatively propagated species, that the stocks are free from soil-borne viruses, but this is more difficult than it sounds, because several species, e g, potato, raspberry, and strawberry, need be propagated on new land for only a year before stocks are distributed to growers, and in this time can become infected without showing symptoms The movement of diseased plants is probably at least partly responsible for the present distribution of raspberry ringspot,

tomato black ring, and arabis mosaic viruses in British soils. It is not safe to assume that stocks propagated on previously uncultivated soil will be virus-free, because tobacco rattle virus, for example, occurs in soils that seem never to have grown crops Probably the best way to propagate stocks would be either to grow them on the same land for at least two years, by which time there will have been a good opportunity for symptoms to develop, or to use land on which a virus-indicator crop has previously been grown With sugarcane, freedom from chlorotic streak disease can be ensured by immersing setts in water at 52°C for 20 minutes before planting (Antoine, 1957).

B. Control Measures on Infested Land

These measures are of four types, all traditionally used to control soil-borne plant pathogens: crop hygiene, rotation of crops, soil treatment, and the use of disease-escaping varieties.

1. Crop Hygiene.

Crop hygiene is of general importance. It includes removing and burning plants infected with tobacco mosaic and tobacco necrosis viruses where this is possible For tobacco mosaic virus (Gardner and Kendrick, 1923) and for other viruses with a wide host range it also includes good weed control

2 Rotation of Crops

Crop rotation is unlikely to free soils of viruses that have a wide host range; it has been shown to be ineffective with raspberry ringspot virus (Cadman, 1956). Avoiding crops that especially favor the build-up in soil of viruses of this type may be worthwhile, for de Bruyn Ouboter and van Slogteren (1949) noted that outbreaks of tulip Augusta disease, caused by tobacco necrosis viruses, were unusually severe on land cropped previously with potato or tobacco, and Quanjer (1926) observed that potato spraing disease tended to be more severe after a turnip crop

By contrast, rotation of crops is very effective in decreasing the incidence of tobacco mosaic and wheat mosaic viruses, and perhaps could eliminate them from soils. Merely alternating tobacco crops with maize controlled tobacco mosaic (Johnson and Ogden, 1929) but a worthwhile degree of control takes longer on land infested with wheat mosaic virus After one crop of potato, rape, buckwheat, or maize, the incidence of wheat mosaic in the next year was still over 90% (McKinney, 1923) but after four years of either oat, soybean, maize, or lucerne crops the incidence of wheat mosaic in the fifth year was 0, 5, 4, and 28%, respectively, compared with 80% on plots cropped each year with susceptible varieties

of wheat The comparatively high figure after weed-infested lucerne is thought to indicate that some of the gramınaceous weeds were hosts of the virus (Koehler, *et al*, 1952).

3 Soil Treatments

Soil treatments of many kinds have proved effective experimentally in freeing soil from viruses, but few have been properly tested or are likely to be economically worthwhile on a large scale For example, steam sterilization of soil probably inactivates all soil-borne viruses but, except for glasshouses or seedbeds, it is unlikely to be an economic proposition.

Some of the many chemicals that have prevented infection by soil-borne viruses when incorporated in infective soil are listed in Table II. Some of these chemicals (e g, formaldehyde) are both virus inactivators and general poisons, others (e g, methyl bromide) are general poisons which have not been tested for ability to inactivate viruses *in vitro* and a few (e g, tetramethylthiuramdisulfide for tomato black ring virus) seem not to inactivate viruses *in vitro* but nevertheless abolish the infectivity of soil Chemicals of the last type are perhaps the most interesting, for they must act on the virus-transmitting system in soil For example, treating soil with tetramethylthiuramdisulfide kills the nematode vector of arabis mosaic virus and prevents plants becoming infected (Harrison and Cadman, 1959), and several nematicides destroy the infectivity of soils containing peach yellow bud mosaic virus (Wagnon and Traylor, 1957), presumably because they kill the vector *Xiphinema americanum*

The few attempts to upset virus-transmitting systems in soil by subtle changes in soil conditions have all failed Thus, the incidence of wheat mosaic virus was not affected by using a wide range of different fertilizers (McKinney, 1923; Koehler *et al*, 1952) However, the observation that increase in amount of potassium fertilizer applied decreased the numbers of *Xiphinema americanum* in a cherry orchard (Kirkpatrick *et al*, 1959) suggests that fertilizers may not be entirely without effect on the incidence of soil-borne viruses

4 Disease-Escaping Varieties

The use of disease-escaping varieties of plants has proved the most successful way of preventing crop losses from soil-borne viruses, and has made it possible to grow economic crops of wheat and oats on land infested with wheat mosaic or oat mosaic viruses (McKinney, 1923; McKinney *et al*, 1949) Varieties that usually show severe symptoms may, however, escape becoming diseased in certain circumstances; for example, late-planted wheat crops are much less severely affected by

TABLE II
CHEMICALS SHOWN TO ABOLISH THE INFECTIVITY OF SOILS

Virus	Chemical*	Reference
Tobacco mosaic	Formaldehyde	McKinney (1927)
Wheat mosaic	Calcium cyanide, carbon disulfide, chloropicrin, D-D, ethanol, formaldehyde, methyl bromide, naphthalene, rotenone	McKinney (1923), Johnson (1945), McKinney *et al* (1957)
Oat mosaic	Chloropicrin, D-D, ethanol, formaldehyde	McKinney *et al* (1957)
Tobacco stunt	Carbon disulfide, chloropicrin, D-D, formaldehyde, mercuric chloride, phenol	Hidaka *et al* (1956)
Grapevine fanleaf (European form)	Carbon disulfide, D-D, mixture of methyl bromide, carbon tetrachloride and dichloroethane	Vuittenez (1957)
Tomato black ring	Ethylene dibromide, formaldehyde, Nemagon, parathion, PCNB, TMTDS	Harrison (1958a), Cadman and Harrison (1960)
Raspberry ringspot	Formaldehyde, parathion	Harrison (1958a)
Arabis mosaic	Ethylene dibromide, PCNB, TMTDS	Harrison and Cadman (1959)
Peach yellow bud mosaic	Carbon disulfide, D-D, methyl bromide, Nemagon, Vapam	Wagnon and Traylor (1957)
Peach rosette mosaic	Chlordane	Fulton and Cation (1959)
Broad bean necrotic mosaic	Chloropicrin	Fujikawa (1955)

* The following abbreviations are used D-D = dichloropropene-dichloropropane, Nemagon = 1,2-dibromo-3-chloropropane, PCNB = pentachloronitrobenzene, TMTDS = tetramethylthiuramdisulfide, Vapam = sodium methyl dithiocarbamate

wheat mosaic than early planted ones (McKinney, 1923), and in Mauritius the incidence of chlorotic streak disease is higher in November-planted sugarcane than in July-planted crops (Antoine, 1957). Varieties that usually escape severe disease may be resistant or immune to infection, may become infected without showing symptoms, or do not become diseased in the field although they show severe symptoms when infected by inoculation of sap. Wheat varieties and wheat mosaic virus illustrate all these types of reaction (McKinney, 1948; Koehler *et al*, 1952).

Immunities to raspberry ringspot, tomato black ring, and arabis mosaic viruses occur independently in different raspberry varieties (Cadman, unpublished observations), even when introduced by grafting with infected scions, the viruses could not be recovered from these varieties Thus there seems a good possibility of breeding a variety immune to all three viruses It is indeed interesting to find, within a species, immunity to viruses to which so many different species, genera, and families of plants are susceptible Disease-escaping varieties are also known in barley to barley yellow mosaic virus (Miyamoto, 1958b), grapevine to the European type of fanleaf virus (Petri, 1937), potato to stem mottle and spraing diseases (both caused by tobacco rattle virus, Noordam, 1956), tobacco to tobacco stunt virus (Hidaka *et al*, 1956) and to tobacco mosaic virus (Holmes, 1954).

VI Conclusions

Perhaps the most important conclusion to be derived from this review is that soil-borne viruses warrant much more attention than they have so far received Many of the diseases they cause are of major economic importance, and their survival in soil and modes of transmission present many fascinating problems The term "soil-borne viruses" as used in this review clearly includes viruses with very different types of interaction with the soil If the present amount of interest in such viruses is maintained, many important advances in knowledge should soon be made, and future reviewers can hope to be able to separate and describe different modes of virus transmission in soil with greater confidence

A second major conclusion is that most soil-borne viruses apparently fall into well-defined groups, although viruses from different groups behave very differently *in vitro*, in plants, and in the soil, some are rod-shaped and others isometric. An attempt has been made in this article to delineate and to characterize some of these groups Soil-borne viruses do not seem to have close affinities with viruses that have aerial vectors, and no virus is known to be transmitted in both ways

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VARIATION IN PLANT VIRUSES

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I Introduction

The purpose of this review is to examine the evidence for the occurrence of strains of plant viruses,† to compare the biological, physical, and chemical properties of the strains, and to examine how they arise Various aspects of variation in plant viruses have been reviewed by Kunkel (1947), Bawden (1950), and Bennett (1953) A more complete review of the problem has recently been made by Knight (1959).

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† The names of the viruses used in this review are from the supplement to Vol 35 (1956) of the Review of Applied Mycology (J C F Hopkins, ed)

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I. Introduction

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* Present address [illegible]

† [illegible]

Among the earliest work which suggested that plant viruses could vary was that of Carsner and Stahl (1924a,b) and Carsner (1925), who showed that sugar beet curly top virus after passage through *Chenopodium murale* produced milder symptoms in sugar beet Johnson (1925) found that tobacco plants infected with a mottling strain of tobacco mosaic virus (TMV) showed an "attenuation" in symptoms after plants were kept at 35°–37°C. for a few days. This change in virulence was maintained when the virus was subcultured in tobacco at ordinary temperatures McKinney (1926, 1929) observed occasional yellow spots on leaves mottled by TMV and showed that virus from the yellow spots produced yellow mosaic symptoms on healthy tobacco plants

Since these discoveries many strains of TMV have been described (e g, J. H. Jensen, 1933) and, indeed, with plant viruses in general, variation seems to be the rule A few have, in the past, been thought of as unvarying, for example, potato leaf roll virus, potato virus Y, and tomato bushy stunt virus However, careful search (Webb *et al.*, 1952; Bawden and Kassanis, 1947; Steere, 1953) or use of a different selection of test plants (Munro, 1955) has usually resulted in the recognition of strains.

II The Technical Problem

In discussing the biological activities of plant viruses certain experimental difficulties must be considered. First, most evidence indicates that very many virus particles must be applied to leaves to cause a single infection Bawden (1950) calculated that about a million particles of TMV are needed to produce one infection Steere (1955), applying inoculum as efficiently as possible, obtained about one infection per 50,000 particles; using tobacco ringspot virus he obtained (Steere, 1956) one infection per 120,000 particles Although Schramm and Engler (1958) claimed to have infected tobacco plants systemically with TMV by applying only about 10 particles per plant, anomalies in the results of these workers (Markham, 1959) suggest that this claim should be accepted with caution. Second, even in a single local lesion many hundreds of cells become infected, presumably after many cycles of virus multiplication, and each infected cell may contain a very large number of virus particles For instance, a cell infected with TMV was calculated (Nixon, 1956) to contain 6×10^7 particles and infection with Rothamsted tobacco necrosis virus may lead to the production of 10^8 particles per cell (Harrison, 1956).

Thus, using current techniques, experiments with plant viruses cannot be made with the precision of similar experiments with bacterial or

animal viruses For example, although it appears entirely reasonable to consider that mutation analogous to that occurring with bacterial and animal viruses also occurs with plant viruses, it is difficult to secure unequivocal evidence for the occurrence of either spontaneous or induced mutation (Section VIII) The possibility that any new strain isolated has been selected from a pre-existing mixture must first be eliminated Further, we cannot tell how many mutations are involved in the formation of a new strain

III Criteria for Identifying Strains

Solution of the problem of relating one virus to others is usually commenced by examining its general properties, such as the symptoms produced in test plants, host range, rate of inactivation on aging or when heated, methods of transmission, and concentration of virus in the sap of diseased plants. Such tests by themselves do not usually have high diagnostic value but they may serve to narrow the scope of further investigation. Because of the large amount of available information on TMV, it is convenient to begin a detailed examination of means of relating strains by reference to this virus A number of criteria have been suggested (e g, Knight, 1950, 1955b) These relate both to the physical and chemical properties of the characteristic particles and to interactions between the virus and its host Strains of TMV· (1) contain similar amounts of ribonucleic acid (Knight, 1954); (2) contain similar proportions of purine and pyrimidine bases in the ribonucleic acid (Markham, 1953), (3) often have the same C-terminal amino-acid residue (Knight, 1955a), (4) are associated with characteristic particles which when studied in the electron microscope appear morphologically similar, (5) give serological cross-reactions (Chester, 1936); (6) have the same order of stability *in vitro;* (7) often exhibit mutual interference (Bennett, 1953); (8) have the same method of transmission; and (9) usually have similar host ranges and produce similar symptoms in some plants.

Although about 300 apparently distinct plant viruses have been described (Smith, 1957), only in a few cases are all the above properties known We must, therefore, discuss the relative importance of the various criteria.

A Chemical Properties

Although all strains of TMV that have been studied contain about 5% ribonucleic acid (Knight and Woody, 1958) so, too, does potato virus X (Bawden, 1950), which has few other properties in common with TMV Thus, the amount of ribonucleic acid cannot alone be used to

relate strains. On the other hand, Markham (1953) suggested that the proportions of the purine and pyrimidine bases may be used as a criterion of relationship.

Harris and Knight (1952, 1955) found that threonine was liberated from TMV protein by carboxypeptidase and Knight (1955a) found that 13 strains of TMV behaved in like manner. Knight (1955b) therefore suggested that the manner of termination of the peptide chains might be useful in classification. The results of Niu *et al.* (1958), who found that the C-terminal amino-acid residues of tomato bushy stunt virus and potato virus X were leucine and proline, respectively, support this idea However, the criterion may be of only limited usefulness as it would exclude from the TMV group cucumber viruses 3 and 4, which have alanine as the C-terminal residue.

Another property which we might include here is that of photoreactivation which is possessed by a wide range of viruses but not by any strain of TMV tested (Bawden and Kleckowski, 1955), although the irradiated free nucleic acid of TMV can be photoreactivated (Bawden and Kleczkowski, 1959)

B Morphology

Although morphology is the chief basis upon which the classification of organisms rests, it has in general been made only very limited use of in comparative plant virus studies.

Particles present in preparations of rod-shaped viruses may differ greatly in length (e g, Bawden and Pirie, 1945; Crook and Sheffield, 1946; Bawden and Crook, 1947; Brakke and Staples, 1958) but appear to be about the same width in the electron microscope The shape and width of virus particles seem to be the same for viruses grouped as related strains by other criteria (Knight, 1959). Bawden and Nixon (1951) examined a number of viruses in crude sap preparations in the electron microscope and were only able to make a broad separation of viruses on the basis of differences in shape and size. Recently an effort has been made to classify viruses with elongated particles according to length of particle (Brandes and Wetter, 1959). The criterion used is the normal length (NL) which is calculated from the main maximum in particle-length distribution curves Most normal lengths tabulated by Brandes and Wetter were calculated from measurements made on preparations obtained using the exudate method of Johnson (1951) or a "dipping" method (Brandes, 1957) They state that "the NL of a given virus prepared from different sources has always been found invariable except for the errors caused by electron microscopy and the technique of measurement" Of the viruses considered, those serologically related

come to be grouped together (e g., potato viruses M and S, and carnation latent virus, NL 650 mμ, type TMV and the bean form of cowpea TMV, NL 300 mμ) However, this criterion also groups together viruses which, although having many properties in common, do not appear to be serologically related (e g, tobacco severe etch virus, henbane mosaic virus, and potato virus Y, NL 730 mμ) Brandes and Wetter conclude that further investigation into the interrelationships of viruses should be preceded by an examination of their morphology, with special consideration of the NL values for viruses with elongated particles

C Serology

Antisera have been prepared to about 32 separate viruses and there seems to be good correlation between degrees of relationship measured serologically and by other means (Matthews, 1957). One difficulty associated with serological methods is that the demonstration of a positive serological reaction depends on the virus titer of the sap For example, a strain present in low concentration may not give a reaction with an antiserum prepared against another strain, despite the fact that the viruses are serologically related (Bawden, 1950)

D Stability

Stability tests are usually made on viruses in unfractioned sap from infected plants The thermal inactivation point and resistance to aging *in vitro* are the measurements most commonly made. There are great ranges in these properties with different plant viruses (Smith, 1957) and they have frequently been used to help in assessing relationship. But many variables are involved in these tests and the methods used have not been standardized Also, as pointed out by Markham (1959), the thermal "inactivation point" of around 55°C observed for many viruses may simply mean that the virus is adsorbed onto cellular material and precipitated with the material at this temperature

E Interference

This test depends upon the observation that when a plant infected with one strain of a virus is inoculated with another strain of the same virus, additional symptoms frequently fail to be produced The results of this type of test have agreed with the results of serological tests applied to potato virus X (Matthews, 1949b) and beet ringspot virus (Harrison, 1958a) The test is, however, not completely reliable as related strains have sometimes been found not to show interference (Bawden and Kassanis, 1951; Kassanis, 1956, Broadbent and Heathcote, 1958). When the two viruses used are unrelated, interference with the

production of systemic symptoms of the second virus has only very occasionally been reported The best-known case is the inhibition of symptom production and multiplication of henbane mosaic virus or potato virus Y in plants already infected with tobacco etch virus (Bawden and Kassanis, 1945). There is no reciprocal protection.

A strain of a virus which interferes with the development of systemic symptoms of another strain will also protect tissue against the formation of local lesions of the second strain. Here, again, degrees of protection have been observed (Matthews, 1949b). This test, however, is much less specific than the first, there being ample evidence of interference between unrelated viruses (Price, 1935; McKinney, 1941; Thomson, 1958).

Working with aster yellows virus strains which protect against one another in plants, Kunkel (1955) has shown that there is also protection in their leafhopper vector Insects which had acquired one strain did not acquire the other strain under conditions that allowed control insects to do so. Experiments to test whether protection in the insect occurred only between related strains were not made. Interference in the vector has also been tested using strains of the "persistent" aphid-transmitted virus of potato leaf roll (Harrison, 1958c). Although plants infected with a mild strain were protected against the severe symptoms typical of another strain, aphids which had transmitted the mild strain were able to acquire and transmit the more severe one at least as efficiently as aphids which were not carrying the mild strain. Harrison pointed out that protection between strains of a virus in the vector may be linked with ability to multiply, for, whereas aster yellows virus has been proved to multiply in leafhoppers (Maramorosch, 1952a, 1956), there is evidence suggesting that potato leaf roll virus does not multiply in aphids (Cadman and Harrison, 1956, Harrison, 1958b). This cannot be taken as decided, however, for the view that potato leaf roll virus does not multiply in aphids conflicts with the results of Stegwee and Ponsen (1958). They found that on several occasions the fifteenth aphid in a group of individuals injected serially with the hemolymph of an aphid carrying potato leaf roll virus, could be made viruliferous. This, they calculate, would be equivalent to a dilution of 10^{-21} of the virus originally present. In direct dilution experiments, however, they find the end point to be 10^{-4}, and they consider that during the serial transfer experiments multiplication in the aphids had probably taken place

It has been suggested that cross-protection in insect vectors might be used to test for relationship between viruses for which this might be the only means at present available (Black, 1959).

Holmes (1956) pointed out that two unrelated viruses infecting the

same plant often produce symptoms which are more severe than either would produce when infecting separately (Bawden, 1935; Bennett, 1949, Rochow and Ross, 1955, Garces-Orejuele and Pound, 1957; Harrison, 1958a). Holmes proposed that this reaction be made the basis of a test of nonrelationship so that, where dual infection is shown to produce an additive effect on symptoms, the two viruses concerned should be regarded as distinct When this test was applied to tomato aspermy virus it was found that additive symptoms were produced in tomato plants also infected with cucumber mosaic virus On the basis of this test the two viruses should be regarded as distinct, although the two have many features in common (Hollings, 1955), and have been regarded as related on the basis of interference and serological tests (Noordam, 1952, Graham, 1957, Govier, 1957) There is the same lack of agreement when Holmes's and other criteria are applied to the viruses of anemone mosaic and cabbage black ringspot Hollings (1957) found that these two serologically related viruses, when mixed in Chinese cabbage, produced symptoms which were much more severe than either produced when infecting alone Caution would therefore seem to be called for in the interpretation of results of the simultaneous infection test, although such results may well provide useful supporting evidence in deciding virus relationships

F. Transmission

The aspect of transmission most likely to be of service in classification is the identification of the vector of insect-transmitted viruses Thus, the only virus transmitted by thrips seems to be that of tomato spotted wilt However, in general the value of this information would be limited to narrowing the scope of further investigation as the aphid- and leaf hopper-transmitted virus groups, for instance, are both very heterogeneous In addition, in at least one instance a virus has been reported to be transmitted by insects from separate groups Thus, both aphids and beetles have been reported to transmit turnip crinkle virus (Martini, 1958).

G Symptoms and Host Range

The host range and symptoms produced on selected plant species have long been known to be unreliable as a basis for relating viruses (e g, Bawden, 1932; Bawden and Sheffield, 1944, Hollings, 1959) Holmes (1958) has suggested the use of a single gene resistance test The basis of this test is that a gene responsible for the resistance of a plant to one virus has not been known to confer resistance to another, unrelated, virus. It is emphasized that this does not imply that a plant resistant

to one strain will necessarily be resistant to all others. He also points out that this test is not the same as the resistant variety test, which has been used in the past as an indication of the relationship between viruses, but which is unreliable because of the possible presence of multigene resistance

II. *Discussion*

Properties of the infective particles and genetic interactions such as recombination would seem to be the most useful sources of information for identifying strains Unfortunately, only about 19 plant viruses have been purified, of which only about 6 have been studied chemically, and no conclusive evidence has been presented of recombination between plant viruses (Section VIII). On the other hand, serological methods can be used with about 32 viruses and, in general, serology has provided the most useful criterion of relationship

IV. Isolation and Maintenance of Strains

A study of variation requires some means of isolating stocks of genetically pure virus. Repeated passage through single local lesions is the method most often used. Evidence of the effectiveness of this procedure comes mainly from experiments using TMV. Kunkel (1934), Lauffer and Price (1945), and Wildman (1959) all found that only a small fraction of lesions obtained by inoculating leaves simultaneously with two strains yielded the two strains on subinoculation When the starting material consists of two unrelated viruses there is evidence that one single lesion passage (Doering *et al*, 1957) or two such passages (Kanarek, 1954) may fail to effect a separation. The probability of obtaining isolates originating from a single particle would obviously be increased by repeated single lesion transfer However, the possibility must be considered that, especially in the case of rod-shaped viruses, polymerization may result in repeated infection with groups of particles. With all such viruses treatments such as precipitation with acids or salts, which may increase the degree of aggregation (Bawden and Kleczkowski, 1948; Kleczkowski and Nixon, 1950), are clearly undesirable. Aggregation of virus particles may also be brought about by allowing sap to age, so that inoculation to the local lesion host should follow immediately upon extraction.

Although the single lesion transfer procedure remains the most useful method of isolating strains, other methods are available. Thus, the use of differential hosts allowed Bawden (1958a) to separate the bean and tobacco forms of the cowpea isolate of TMV and Bagnall *et al* (1956, 1959) were able to separate potato virus M from a mixture containing

potato virus S by passage through tomato or Saco potato Holmes (1934) obtained a strain of TMV producing very mild symptoms on tobacco by passing a virulent isolate through plants at 34°C. This procedure may have selected a strain which could infect at this temperature (Kassanis, 1957) and presumably would be useful whenever strains differ in their ability to infect and multiply at high temperature (Hitchborn, 1956, 1957).

Even when it is reasonably certain that a stock of virus was originally genetically pure, maintaining it in this condition presents a major problem Assuming that the mutation rate of plant viruses is about the same as that of bacterial viruses, then many mutants are likely to occur even in a single local lesion Recognition of the huge populations of virus particles in systemically infected plants leads to the conclusion that it is improbable that an isolate will remain genetically homogeneous for long. Perhaps the most of which we can be sure is that the isolate contains particles predominantly of a certain strain or that less than a certain proportion of contaminating particles is present

V Frequency of Mutation

Little is known about the mutation rate of plant viruses and concerns only mutants which induce symptoms different from those induced by the parent strain Present information suggests that all viruses do not give rise equally readily to recognizable mutants Thus, while dozens of mutants of TMV have been isolated only a few strains of tomato bushy stunt virus have been recognized (see Section I) It has also been suggested that different strains of potato virus X may vary in their mutability (Matthews, 1949a). Similarly, Fulton (1952) found that strains of tobacco necrosis virus which produced white lesions on cowpea frequently gave rise to strains giving red lesions but that the reverse change was not seen to occur Fulton emphasized, however, that this cannot be taken as proof of a difference in mutability between the strains producing red lesions and those producing white lesions, as it may merely mean that the former dominated when the two types were in competition

VI Differences between Strains

We have already discussed the properties common to plant virus strains We shall now examine the various differences that may occur between strains.

A Chemical

Information on the kinds of chemical differences between strains of plant viruses comes mainly from studies on TMV Strains of most other

viruses have not been purified sufficiently for chemical analysis. Chemical studies have, however, been made on strains of tomato bushy stunt virus (de Fremery and Knight, 1955) and potato virus X (Markham, 1953). Differences between strains in respect of the chemical composition of the protein and of the nucleic acid moieties will be considered separately.

1 The Protein

The first complete analyses of protein from six strains of TMV were made by Knight (1947) Later, seven other strains were studied (Black and Knight, 1953). In these studies microbiological methods were mainly used. The most important conclusions (Knight, 1959) from this work were: (1) *definite differences in protein composition occur between strains* (2) These differences were mainly in the proportion of amino acids but differences in type also occurred Thus, histidine and methionine were present in the rib grass (RB) strain (Holmes, 1941) but not in the type strain. (3) Differences in composition were not confined to certain amino acids, for in 13 strains differences affecting 15 out of 18 amino acids occurred Only cysteine, leucine, and proline appeared constant in all strains (4) The M (Holmes, 1934) and type strains of TMV showed no detectable differences in protein composition yet they could be readily distinguished by the type of symptoms they produced. (5) Strains closely related in their derivation were similar in composition.

The findings of Bawden (1958a) on the cowpea strain of TMV suggest that this last conclusion of Knight's may require revision. There is evidence that the tobacco form of this virus mutates to produce the bean form and vice versa, the change taking place during a single host passage. Although, as Bawden points out, there is no indication of the number of mutations involved, the emergence of one form from the other during a relatively short space of time would normally lead to their being regarded as closely related There are, however, relatively large differences between the amino acid compositions of the two forms.

Structural studies on the protein from TMV have been made. Knight (1955a) *found that 13 strains of TMV had threonine as the C-terminal* amino acid residue Approximately the same amount of threonine was obtained from each strain On the other hand, alanine has been found to be the C-terminal amino acid residue of cucumber viruses 3 and 4 (Niu *et al*, 1958), which are serologically related to TMV. A study (Niu and Fraenkel-Conrat, 1955) of C-terminal hexapeptides of 4 strains of TMV showed that the amino acid sequences of those from the type, M, and YA strains were identical, but that the RB strain yielded a

hexapeptide with amino acids in somewhat different sequence Knight (1957) found that TMV protein treated with trypsin yielded 13 peptides The peptide patterns of the type and M strains of TMV appeared to be indistinguishable On the other hand, the patterns of RB and type TMV were widely different and that of YA was similar to, but not identical with, that of type TMV.

2 The Nucleic Acid

As already noted (Section III), all strains of one virus seem to have about the same proportion of nucleic acid In addition, they have frequently been found to contain similar proportions of purine and pyrimidine bases, in contrast to the proportions for distinct viruses However, there are known exceptions to this generalization, for cucumber viruses 3 and 4 have base proportions somewhat different from those of other TMV strains analyzed It has even been questioned whether a specific ribonucleic acid composition can be associated with a particular strain. Using isolates of common TMV which had been passed through single local lesions several times, Commoner and Basler (1956) made analyses of the total nucleic acid content and of the "molar proportions of the individual nitrogen bases" of a large number of purified preparations which electrophoretic analysis showed to be homogeneous They found that, to a high degree of probability, both varied with the length of time for which infection had been allowed to proceed and with the physiological condition of the tissue from which they were extracted They suggest as probable that "no specific values for nucleic acid content or nitrogen base proportions could be regarded as characteristic of the common strain" It is pointed out by Markham (1959), however, that there is some evidence that, as used, the method was not sufficiently quantitative to support this conclusion

The arrangement of the nucleotides in the polynucleotide chains has been found to differentiate between strains which have nucleic acids of similar composition Thus, the M strain of TMV has been distinguished from the type, RB, and YA strains on this basis (Reddi, 1957), and in later work on the type, RB, and M strains, evidence was obtained (Reddi, 1959) of a specific arrangement of the nucleotides in each strain

B. Physicochemical

Purified preparations of strains of TMV have been reported to differ in a number of respects These include electrophoretic mobility (Friedrich-Freksa *et al*, 1946; Kahler and Woods, 1949; Singer *et al*, 1951, Siegel and Wildman, 1954; Bawden, 1958a) and isoelectric point

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(Oster, 1951, Gordon and Price, 1953). Both of these properties depend on the character of the surface of the particles and, therefore, probably reflect differences between the surfaces of the proteins of the strains The U2 and U1 strains of TMV (Siegel and Wildman, 1954) also differ from each other in that the nucleic acid of the U2 strain is released more easily from the nucleoprotein by the heat-detergent method than is that of the U1 strain, and in that heat denaturation of the U2 strain takes place more readily than does that of the U1 strain (Siegel *et al.*, 1956). Strains of TMV have also been found to differ in their ability to combine with host nucleoprotein, which, when purification was carried out in the presence of cacodylic acid buffer, determined whether the preparation was brownish or colorless (Ginoza *et al*, 1954; Siegel and Wildman, 1954). Several means could be used to decolorize such preparations, but the specific ability to become colored remained.

C Morphological

Electron microscope studies of plant viruses have provided little evidence of differences in shape and size between strains of the same virus (Section III) Bawden (1958a), however, states that the bean form of the cowpea strain of TMV produces particles which are shorter than those of the tobacco form The electron microscope evidence was supported by the electrophoretic and serological behavior of the two forms. Electron microscope measurements made by Brandes and Wetter (1959), however, failed to confirm Bawden's observation.

X-ray diffraction studies made by Bernal and Fankuchen (1941) and Franklin (1956) on cucumber virus 4 and three other strains of TMV showed that the packing diameter of the cucumber virus particles was about 3% less than that of particles of the other strains of TMV Measurement of the radial electron density distribution of particles made by Holmes and Franklin (1958) has demonstrated that the difference lies not between the maximum diameters of the particles of the strains but between the depths to which the particles can interlock. These workers studied the U1 and U2 strains (Siegel and Wildman, 1954) and the bean and tobacco forms of the cowpea strain of TMV (Bawden, 1958a) and cucumber virus 4 They found that the particles of all the strains had a hollow core of radius about 20 Å, a maximum radius of about 90 Å, a high density peak due to nucleic acid at a radius of 40 Å, and prominent peaks in protein density at 24, 66, and 78 Å There were, however, differences in the heights of the protein peaks For example, the bean form and the tobacco form of the cowpea TMV could be readily distinguished on this basis

D. Biological

1. Symptoms and Host Range

Investigation into the relationship that an unidentified virus bears to other viruses is usually begun by observing the symptoms which it produces in a variety of host plants. This recognizes the general principle that strains of a virus tend to produce similar symptoms on any particular host Thus, a great number of strains of TMV, for instance, produce characteristic mottles on tobacco and only necrotic local lesions on *Nicotiana glutinosa* at 22°C. However, early in the study of plant viruses it was noticed that isolates resembling each other closely in features such as stability, concentration in the sap of infected plants, host range, means of transmission, and in the symptoms produced in infected plants, nevertheless differed slightly in this latter respect It is now recognized that one of the commonest variations between strains is the different symptoms they produce in infected plants Moreover, it is the most sensitive, and often the only, means of distinguishing between strains Some of the differences between the reactions excited in a host species by different strains of the same virus are large For instance, TMV produces only pinpoint local lesions on inoculated cucumber leaves, whereas cucumber viruses 3 and 4 produce systemic mottling in cucumber, and a similar difference exists in the response of cowpea to different strains of cucumber mosaic virus (Price, 1934, Fulton, 1950)

All strains of a virus do not always have the same host range Thus, cucumber viruses 3 and 4 do not appear to multiply in tobacco or *N. glutinosa*, which are hosts of other strains of TMV, of these latter strains only the U2 strain seems unable to infect Kondine Red tomato (Bawden and Pirie, 1956) Matthews (1949a), Bercks (1953), and Rochow and Ross (1955) reported that while certain isolates of potato virus X were able to infect potato, others were not, Thomson (1956) isolated a strain of potato virus X which apparently did not infect *Chenopodium album*, although a number of other strains did A virus from *Tropaeolum* (Smith, 1950), which we have shown to be serologically related to cabbage black ringspot, readily infects *Tropaeolum*, although repeated attempts to infect this plant with other strains of cabbage black ringspot all failed California aster yellows virus will readily infect celery but the eastern form of the virus is much more difficult to transmit to this host (Kunkel, 1955)

2 Transmission

It is well known that while some strains of a virus may be transmissible by an insect species, other strains may not be A number of

instances of loss of ability to be transmitted while an isolate was under observation have been reported (Black, 1953; Hollings, 1955; Watson, 1956, Swenson, 1957; Badami, 1958; Black *et al.*, 1958). There are also instances of strains in the field having specific insect vectors. Thus, the New York and New Jersey yellow dwarf viruses of potato, which are considered to be related, are spread by different leaf hopper species (Black, 1941). Similarly, the Argentine and North American sugar beet curly top viruses have different vectors (Bennett *et al*, 1946). However, there is scant evidence for regarding these two viruses as related. The high degree of specificity in the transmission of viruses by insects cannot be explained with certainty at present; however, for those viruses which multiply in their vectors, it has been suggested (Maramorosch, 1955) that specificity may be correlated with ability to multiply. Support for this idea comes from the results of Maramorosch (1952b, 1955), who found that aster yellows virus multiplied in vector species but not in non-vectors Similarly, Black *et al* (1958) found that a vectorless strain of wound tumor virus apparently did not multiply in a leaf hopper species which supported the multiplication and acted as vector of another strain With nonpersistent, aphid-transmitted viruses there is evidence that the concentration of virus in the plant influences the frequency of transmission For instance, Bhargava (1951) found that the No. 6 yellow strain of cucumber mosaic virus (Price, 1934), which was transmitted relatively inefficiently by *Myzus persicae*, was in lower concentration than other more efficiently transmitted strains Bhargava thought, however, that other factors were concerned and there is little doubt that differences in the efficiency with which strains are transmitted cannot be explained on the basis of differences in concentration of virus alone It has been shown, for instance (Hitchborn, 1958), that a form of potato virus C which could not be transmitted by *M. persicae* was present in systemically infected leaves in about as high a concentration, in both the epidermis and the leaf lamina as a whole, as a strain of the serologically related potato virus Y, which was very efficiently transmitted

3. Sensitivity to Inactivation

Strains have frequently been reported to vary in their resistance to aging and to heat inactivation Such reports are concerned chiefly with measurements made using unfractioned infective sap where differences other than intrinsic ones between the strains could influence results For example, pH influences the rate of inactivation of TMV at high temperatures (Bawden, 1950), and its denaturation by heat has been shown to be affected by pH and by concentration of virus (Lauffer and Price, 1940).

Differences in the sensitivity to inactivation by ultraviolet irradiation have been described by Siegel and Wildman (1954) and by Bawden (1958a). Siegel and Wildman found that the more resistant strains were those which gave brownish preparations, but it was shown that there was no causal relationship between color and resistance to irradiation, inasmuch as removal of the color did not affect resistance. Further studies on two of these strains, namely, U1 and U2, which differ by a factor of 5½ in their sensitivity to inactivation (Siegel and Wildman, 1956), have shown that the free nucleic acids of the two strains have the same sensitivity and that this is the same as that of the more sensitive strain (Siegel *et al*, 1956). Reconstitution of the strain that was more resistant when intact did not restore this resistance, suggesting that the protein did not confer resistance by screening the nucleic acid from irradiation, but by means of specific bonding with it. Drying the two strains U1 and U2 abolishes the difference in sensitivity between them (Siegel, 1957). Further study did not support the hypothesis that the difference was due to a difference in the degree of hydration of the two strains.

The extent to which high temperature affects different strains *in vivo* also varies (Holmes, 1934, Hitchborn, 1956, 1957). By keeping infected plants at about 37°C, Hitchborn found that some strains of cucumber mosaic virus and a strain of tobacco ringspot virus could be eliminated, and these strains did not become established in inoculated plants at this temperature (cf Kassanis, 1952, 1954). In contrast, other strains were not eliminated from infected plants by the treatment and could infect healthy plants at this temperature.

4. The Infection Process

Information on the progress of the infection process immediately after inoculation has been obtained by measurement of the sensitivity of infections to inactivation by ultraviolet light. Differences between strains have been found in the rate at which changes in sensitivity occur after inoculation. Siegel and Wildman (1956) and Siegel *et al* (1957) found that at 20°C the infectious centers of the U2 strain of TMV increased in resistance after 2 hours' incubation, but that those of the U1 strain did not until after 5 hours' incubation. Infections initiated by using the nucleic acid of the two strains increased in resistance to inactivation without an appreciable time lag. It was suggested that when intact virus is inoculated the nucleic acid of the particles remains bound to protein for several hours and that this time may be longer for the U1 than for the U2 strain. The change in sensitivity with time of different strains of potato virus Y is also different (Hitchborn, 1959). Study of

two strains showed that, in contrast to the U1 and U2 strains of TMV and to Rothamsted tobacco necrosis virus (Bawden and Harrison, 1955), both strains of potato virus Y became increasingly sensitive to inactivation for the first 2½ to 3 hours after inoculation at 22°C They differed from each other, however, in that the increase was greater with strain A than with strain B. Moreover, decrease in sensitivity, which began at 2½ hours with strain A, did not begin until 5 hours after inoculation with strain B.

5 Specific Infectivity

The infectivity per unit weight of purified type TMV was consistently found to be greater than that of a *Datura* strain when both were inoculated in *N. glutinosa* (Bawden and Pirie, 1956). Measured on the basis of either weight of virus per unit volume of purified preparation or serological activity of sap from systemically infected plants, the tobacco form of the cowpea strain of TMV was about 10 times more infectious in *N. glutinosa* than was the bean form (Bawden, 1958a). The rib grass and green mosaic strains of TMV also differ in their infectivity per unit weight; Fraenkel-Conrat and Singer (1957) have given evidence that this difference is associated with the protein. First, the free nucleic acids of the two strains have the same specific infectivity Second, if the protein of the green mosaic strain, which is the more infectious strain, is combined with the nucleic acid of the rib grass strain, the specific infectivity of the reconstituted virus is greater than that of the original rib grass strain.

VII. Detection and Estimation of Strains in Mixtures

Bawden (1956, 1958a) found that the tobacco and bean forms of cowpea TMV produced dissimilar, characteristic lesions on *N. glutinosa;* he used the distinction to make rough quantitative estimates of the amounts of the tobacco form in mixtures He found that the number of lesions of the tobacco form was little affected by varying the amounts of the bean form present There is, however, much evidence that the number of lesions produced by one strain can be affected by the presence in the inoculum of another strain of the same virus This has been shown for potato virus X and tomato mosaic virus (Sadasivan, 1940), TMV (Mundry, 1957; Siegel, 1959; Bawden, 1959), and potato virus Y (Thomson, 1959). Such interference could clearly limit the usefulness of the method However, the degree of interference depends not only on the relative amounts of the two strains present but also on the absolute amounts, becoming less the lower the concentration (Mundry, 1957; Siegel, 1959). Thus, accurate assay may be possible by this method,

even when the two strains can interfere with one another, provided the assay is done at a sufficiently low virus concentration

Differences in the electrophoretic mobility of strains have been used as the basis for the quantitative estimation of two strains of tobacco mosaic virus (Zaitlin, 1956, Cohen *et al*, 1957, Bawden, 1958a).

In addition to antigens which two strains may have in common, there may also be antigens specific to one or each of the strains Where large differences between strains occur, the cross-absorption procedure can be used for detecting the presence of strains which have antigens not shared by other strains present in a mixture. Under suitable conditions of antibody and antigen concentration it has also been shown that there are two α-optima when two strains of TMV are precipitated from a mixture, even when the two are closely related serologically (Matthews, 1957)

VIII Origin of Strains

Strains have been obtained by a number of methods As already discussed (Section II), it is difficult with present techniques to decide whether a newly isolated strain arose during an experiment by, for example, mutation, or whether it was present in an original mixture and was selected.

A *Spontaneous Appearance*

Among the earliest studies on strains were those on the spectacular yellow spots appearing on leaves of plants infected with strains of TMV (Jensen, 1933) and cucumber mosaic virus (Price, 1934), which produced mainly a green mottle on infected plants Inoculation from such spots onto healthy plants often produced a disease in which yellowing was the predominant feature Experimental evidence was presented in support of the idea that such spots arose as a result of the local multiplication of a mutant rather than of a minor constituent of the original virus stock For instance, passage of the green mottle stock of TMV through a series of single lesions in *N glutinosa* in an attempt to eliminate any such minor fraction failed to prevent the appearance of yellow spots on infected tobacco plants This in spite of the fact that the yellow strains were difficult to subculture in *N. glutinosa*

B *Growth in Different Hosts*

There are several reports of the virulence of viruses being changed by passage through one of their hosts Carsner and Stahl (1924a,b) showed that sugar beet curly top virus, after growth in *Chenopodium murale*, produced milder symptoms in sugar beet than originally Changes in the symptoms produced by potato virus X after growth in certain hosts

were described by Salaman (1939), a change in strain type from severe to mild occurring after growth in sugar beet or red beet. The change could also apparently be brought about by mixing the virus with sugar beet sap *in vitro*.

Johnson (1947) found that when a culture of TMV which produced severe symptoms in tobacco was inoculated in sea holly (*Eryngium aquaticum*) and subcultured from the systemically infected leaves, the virus obtained produced mild symptoms in tobacco. It was shown that sea holly produced this change by selecting a mild strain from an original mixture which also contained a strain that gave only local lesions in this plant. Similarly, the changes in severity of potato virus X stocks, which Matthews (1949c) found followed growth in *Cyphomandra betacea*, were attributed to the selection of certain strains from a pre-existing mixture.

Host-induced changes in the infectivity of a virus for a certain host have also been reported Thus, potato virus X grown in a nonpotato host has been shown to lose its ability to infect potato (Matthews, 1949a; Bercks, 1953; Rochow and Ross, 1955). Yarwood (1953) states that when a tobacco necrosis virus was maintained in bean it gradually became less infectious for tobacco. Reduction in invasiveness of a virus for one host when grown in another host has been reported for a tobacco necrosis virus (Yarwood, 1953), potato virus Y (Marcus, 1953), and lucerne mosaic virus (Oswald *et al*, 1955).

Another host-induced change in a virus has been described by Watson (1956). A stock of potato virus C which was aphid transmissible often became nontransmissible after passage through potato.

Changes brought about by passage through certain host species have sometimes been shown to be reversible by passage through another species For instance, the attenuation of sugar beet curly top virus after passage through *Chenopodium murale* (Carsner and Stahl, 1924a,b) was reported to be reversed by passage through *Stellaria media* (Lackey, 1931). Evidence for similar reversal of host-induced changes has been given for potato stem mottle virus (Köhler, 1956) and potato virus X (Thomson, 1956).

In the above cases no evidence was provided of changes in the physicochemical properties of the virus. Bawden (1956, 1958a) has, however, studied reversible changes in these properties in a strain of TMV from cowpea. The cowpea strain of TMV, when purified from French bean (*Phaseolus vulgaris* var. Prince) and tobacco, differed in physicochemical properties as well as symptoms produced in host plants Furthermore, the bean form when inoculated in tobacco led to systemic infection by the tobacco form and the tobacco form inoculated in bean

resulted in systemic infection by the bean form Some of the differences between the tobacco and bean forms observed by Bawden were in: (1) sensitivity to inactivation by ultraviolet irradiation; (2) particle size, (3) specific infectivity, (4) electrophoretic mobility; (5) serology; and (6) protein composition: for instance, the protein of the bean form contained histidine, whereas that of the tobacco form did not

These host-induced changes of the virus from cowpea could be due to (a) the presence of both forms in the inocula and selection by bean and tobacco of the respective forms, (b) the selection of randomly occurring mutants, (c) the selection of host-induced mutants, (d) the production of phenotypic variants (Bawden, 1956)

The hypothesis of phenotypic variation involved the idea that the nucleic acid of the two forms was the same and became combined with different proteins in different hosts Strong evidence against this was obtained (Bawden, 1958a) when it was found that the nucleic acids of the two strains gave local lesions on *N glutinosa* characteristic of the forms from which they were derived

Although the possibility exists that the results could be due to failure to separate from each other two strains present in the original material, Bawden (1958a) gives reasons for believing this unlikely These included the fact that after four single lesion passages in *N glutinosa* each form reverted to the other on passage through the appropriate host During passage in *N glutinosa* each form produced only its own type of lesion As the tobacco form is much more infectious in *N glutinosa* sap than the bean form, it was thought especially unlikely that the bean form could be contaminated with the tobacco form in this host without its being detected

It was pointed out by Bawden that although it was not possible to decide between the hypothesis of selection of randomly occurring mutants and that of selection of mutants induced by the host, most of the results could satisfactorily be explained in terms of the first hypothesis

C Growth at High Temperature

Holmes (1934) first showed that a "masked" strain of TMV could be obtained when tissue infected with a type strain of TMV was kept at 34°C This phenomenon has been confirmed by Johnson (1947) and Sukhov (1956). These changes occurred after the original inoculum had been passed through a series of single lesion transfers

D Physical and Chemical Treatments

With animal and bacterial viruses there are many instances of mutation induced by chemical and radiation treatment Although a number

of such treatments have been shown to alter the character of plant virus suspensions or cultures, evidence that mutation has been induced is generally not good. Gowen (1941) found that after X-ray irradiation *in vitro*, type TMV produced more necrotic lesions on tobacco than did untreated virus and that the necrotic lesion-producing TMV yielded type TMV more often after irradiation. The effect increased with increasing X-ray dose. Gowen attributed the change to mutations brought about by the treatment. Smith (1951) states that a necrotic-reacting strain of TMV was isolated after irradiating type TMV with X-rays. Mundry (1957) has reinvestigated the possibility of producing mutants by irradiating TMV *in vitro* with X-rays and with ultraviolet light. His results do not provide evidence that such treatments were mutagenic. Few attempts appear to have been made to produce mutants by irradiating leaves in which virus is multiplying. Kausche and Stubbe (1940) did, however, conclude that although mutants of TMV were not produced by treating the virus *in vitro* with X-rays or gamma rays, the mutation rate was increased by irradiating infected plants. Larson *et. al* (1955) isolated new strains from leaves of *Nicotiana rustica* infected with potato virus X and treated with nitrogen mustard. Although the isolate of potato virus X used had never been seen to vary spontaneously it was agreed that selection was not excluded.

The nucleic acid of plant viruses seems to be the infective material (Fraenkel-Conrat, 1956, Gierer and Schramm, 1956). It is reasonable to suppose, therefore, that mutations are more likely to be induced by altering the nucleic acid than by altering the protein of the virus (Knight, 1959). It is of interest to note here that the pyrimidine analog, 5-bromouracil, which can be incorporated into phage deoxyribonucleic acid (Dunn and Smith, 1957), is mutagenic for phage (Litman and Pardee, 1956). It has been shown that 8-azaguanine (Matthews, 1954, 1955), 2-thiouracil (Jeener and Rosseels, 1953, Matthews, 1956), and 5-fluorouracil (Gordon and Staehelin, 1958) are incorporated into the nucleic acid of TMV or turnip yellow mosaic virus. This incorporation may result in the production of noninfective particles (Matthews, 1955, Francki and Matthews, 1959), but little work has been done on the possibility that the incorporation of the unnatural base results in nonlethal mutation. Bawden and Kassanis (1954) did find that broad bean mottle virus and a strain of TMV, which did not produce lesions on untreated leaves, produced lesions on leaves treated with thiouracil. However, the possibility that the effect of the thiouracil was due to genetic changes in the viruses and not simply to changes in the host-plant response to infection does not seem to have been examined. Thomson (1959) failed to demonstrate changes in strains of TMV and potato virus X after treating infected

plants with thiouracil, although the treatment greatly affected the types of lesion produced.

Gierer and Mundry have presented evidence for the production of mutations in TMV by nitrous acid (Gierer and Mundry, 1958, Mundry and Gierer, 1958) They used a strain of TMV which produced on tobacco var. Java predominantly faint chlorotic lesions with a few necrotic lesions. They found that the number of necrotic lesions could be progressively increased by increasing the length of time the virus nucleic acid was treated with nitrous acid It was concluded that the treatment was mutagenic Bawden (1959) confirmed these results but did not accept that the effect was necessarily due to induced mutation He pointed out that in his experiments and in those of Gierer and Mundry the starting material consisted of a mixture of strains which produced both chlorotic and necrotic local lesions, and he argued that selection could not be ruled out as an explanation of the effect of nitrous acid. The case against selection as a possible mechanism has been put by Mundry (1959). It is noteworthy that Boeyé (1959), who found that treatment of poliovirus with nitrous acid increased the proportion of a certain mutant type in the surviving virus, obtained evidence that this was not the result of selection Boeyé concluded that nitrous acid was mutagenic for poliovirus.

E. Interaction between Related or Unrelated Viruses

Genetic recombination between bacterial viruses and between animal viruses has been shown to occur. Critical examination of the possibility that recombination takes place between plant viruses is made difficult by the relatively crude methods available for their study Moreover, multiple infection of single cells is presumably a prerequisite of recombination and there is some doubt that this can take place Thus, Siegel and Wildman (1956) found that when the concentration of TMV in inoculum was increased so that the approximate mean number of infectious units per infection was increased from 1 to 7, the ultraviolet survival curve remained exponential This was taken as evidence that for single strain inoculation at least, only one unit took part in each infection, even when several were present Using mixtures of the U1 and U2 strains of TMV, Siegel (1959) inoculated leaves of *Nicotiana sylvestris* in which only the U2 strain produces necrotic local lesions The presence of the U1 strain above a certain concentration in the inoculum reduced the number of necrotic lesions produced From an examination of the results Siegel concluded that where a susceptible site on a leaf is presented with particles of more than one strain, only one of these particles is usually able to infect However, the assumption

seems to have been made that a lesion initiated by both U2 and U1 particles will be necrotic. Siegel showed that the number of lesions of U2 was unaffected by heavy inoculation with U1 an hour before or after inoculation with U2, which suggests that interference between developing lesions of the two strains was not important. However, this does not seem to preclude the possibility that where the U1 and U2 strains were inoculated together, both strains could infect the same cell and the necrotic symptoms typical of U2 fail to develop (cf. Benda, 1959). Evidence for multiple infection comes from the experiments of Lauffer and Price (1945), who found that simultaneous inoculation with two strains of TMV led to the formation of some lesions which contained both strains, although the incidence of such lesions was much lower than had been expected from theoretical considerations Kunkel (1934) and Wildman (1959) also found that inoculation with a mixture of two strains of TMV led to the production of some lesions which contained both strains Wildman, however, emphasizes that a single lesion may arise from the union of two infections originating in separate cells. It is clear from the experiments of Benda (1956) that two strains of TMV can be injected into a single leaf cell and both subsequently multiply in the leaf As Benda points out, however, his results do not prove that multiplication of the two strains had occurred in the same cell, since in the 25% of lesions which contained both strains the two may have been multiplying in neighboring, but separate, cells. Sukhov (1956) found that the two types of inclusion bodies characteristic of two strains of TMV could be present in the same cell This suggests that these strains could multiply in the same cell.

Although evidence is lacking that mixed infection of single cells commonly occurs, there are reports of new strains being isolated from plants infected with more than one strain of tomato spotted wilt virus (Best, 1954, 1957, Best and Gallus, 1955), TMV (Sukhov, 1956), and potato virus X (Thomson, 1959). Best and Gallus found that from plants infected with two strains of tomato spotted wilt virus other strains could be isolated which, with regard to symptoms produced in various hosts, combined certain characteristics specific to each of the original strains New strains have also been reported in "reconstitution" experiments with TMV protein and nucleic acid (Commoner *et al*, 1956; Fraenkel-Conrat and Singer, 1957), but no evidence of the genetic purity of the original inocula was presented.

Thomson (1959) has shown that the type of local lesion produced in leaves by a virus may be modified if the leaves are infected with another unrelated virus No evidence was obtained that this was due to a genetic change in the virus. However, Sukhov and Kapitza (1956)

found that a strain of potato virus X, not previously met with, was obtained from tobacco plants infected with both potato virus X and TMV.

IX ECOLOGY AND ECONOMIC IMPORTANCE

In the field selection against strains which reduce the vigor or life span of their hosts will tend to occur naturally, although this tendency will be reduced if such strains have alternative tolerant hosts (Bawden, 1950). Roguing, also, will operate to the advantage of mild strains King Edward potatoes infected with paracrinkle virus are almost completely symptomless and so are many potato varieties infected with potato virus S This has been attributed by Bawden (1958b) to the application of roguing together with the absence of a natural means of spread of these strains Intensive roguing may also explain why many potato varieties on an Arran (Scotland) breeding estate were found to be 100% infected with strains of potato virus X which produced no symptoms (Matthews, 1949b)

There are a number of reports of isolates of virus losing their original insect transmissibility during maintenance without insect transmission (Black, 1953; Hollings, 1955, Swenson, 1957, Black *et al*, 1958, Jensen, 1959) Under these conditions a premium would be put on a high rate of multiplication and invasion and if a mutant arose which possessed these properties to a higher degree than the parent strain, it might replace the latter even though it lacked the ability to be insect-transmitted This process has been envisaged as operating in the field, resulting in the nontransmissibility by insects of viruses in plants normally vegetatively propagated This explanation has been applied to potato viruses C and S and to paracrinkle virus (Black, 1953, Bawden, 1958b), none of which are insect transmitted, although they are related to viruses that are Alternative hypotheses have, however, been put forward to explain how a virus, lacking the survival advantage of insect transmissibility possessed by related viruses, may occur widely Bawden and Kassanis (1947) favored the view that potato virus C was at one time aphid-transmitted in the field but that its vector lost the capacity to transmit Watson (1956) found that the Rothamsted stock of potato virus C, which had been maintained in tobacco and *N glutinosa* for a number of years and which was aphid transmissible, usually lost this capacity after passage through potato She suggested, therefore, that potato virus C may occur in nature in an aphid transmissible form in a solanaceous weed, from which infection of potato could occur. In this connection the discovery by Badami and Kassanis (1959) of an aphid transmissible form of potato virus C in a plant of *Solanum jasminoides*

from India is interesting, although this virus apparently retained its aphid transmissibility after passage through potato.

Only a little is known about the distribution of strains and the relationship between strains isolated from different sources. Johnson and Valleau (1946) noted that a different dominant strain of TMV tended to characterize different farms in old tobacco growing areas This is reasonably explained by supposing that the dominant strain is present between crops of tobacco either in alternative hosts or on clothes or implements. There may be a similar explanation for the common occurrence in greenhouse tomato crops, in England, of strains of TMV which produce only a hypersensitive response in tobacco. However, the maintenance of these strains in their dominant position in the face of competition from strains which are present in smoking tobacco and which presumably usually become systemic in tobacco suggests that other factors may be involved Matthews (1949b) found some evidence for a higher degree of serological relationship between strains of potato virus X from the same source than between strains from different sources Evidence of increasing divergence of strains with increasing geographical separation comes from the work of Harrison (1958a,d) on two soil-transmitted viruses He found that, among strains of tomato black ring virus, which includes the viruses of potato bouquet and beet ringspot, the degree of serological relationship between strains was greater the smaller the distance separating the areas from which the strains came There were similar findings when a number of isolates of raspberry ringspot virus were examined

X Prospects

Bacterial viruses have provided "model" systems for the study of virus growth and interaction phenomena such as recombination. Although knowledge of the chemistry and physics of some plant viruses has accumulated rapidly—a process helped by the relatively large amounts of stable, easily purified virus available—advance in the biological field has been much slower Some of the reasons why studies in the genetics of plant viruses cannot be made with the precision of similar studies of bacterial viruses have been discussed (Section II). Even with the most favorable host-virus systems unequivocal results often cannot be obtained.

One technical improvement would be the use of single cell systems Progress has recently been made in this direction by the isolation of apparently undamaged plant cells after fragmenting leaf tissue with pectinase (Zaitlin, 1959), and Bergmann (1959) has isolated cells, which will divide when suspended in agar, by shaking cultured, undifferentiated

callus tissue in liquid media. Unfortunately, present evidence indicates that intact, cultured cells cannot be infected by plant viruses (Kassanis *et al*, 1958; Bergmann and Melchers, 1959). However, the possibility remains that the cell walls can be degraded in some way and the cells made susceptible.

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DENSITY GRADIENT CENTRIFUGATION AND ITS APPLICATION TO PLANT VIRUSES*

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I Introduction

Density gradient centrifugation refers to techniques wherein particles are centrifuged through a liquid column which has a density gradient resulting from concentration gradients of two other substances of different densities The gradient column is in a centrifuge tube If the particles are plant viruses, the gradient column could be a sucrose

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solution with a positive sucrose-concentration gradient and a negative water-concentration gradient.† If the particles are present throughout the column before centrifugation, they will be found after centrifugation in a zone where the density of the supporting medium is equal to that of the particles. If the particles are floated in a layer on top of the column, they will sediment as a zone through the column during centrifugation and eventually reach a position of equal density. The centrifuge may be stopped at any time before this, and the particles will be in a stable zone at a depth dependent on their properties.

The numerous possible modifications of this method will be classified in three categories. The term "isopycnic gradient centrifugation" (Anderson, 1955) will be used for methods in which centrifugation is continued until each particle has reached a point where the suspending medium has a density equal to that of the particle. The particles may be distributed throughout the column, or in a layer at the top of the column, before centrifugation. The supporting concentration gradient may be formed prior to or during centrifugation (Meselson *et al*, 1957). Isopycnic gradient centrifugation separates particles on the basis of their densities and may also be used for the determination of densities.

The other two categories of methods will be called "rate zonal centrifugation" and "equilibrium zonal centrifugation." In these the virus solution is floated in a layer on top of a preformed gradient column before centrifugation. Each type of particle sediments as a zone through the column at a rate dependent on its size, shape, and density. If the centrifuge is stopped while the virus is still sedimenting rapidly, the technique will be termed "rate zonal centrifugation" (Brakke, 1956). Particles are separated on basis of their sedimentation rates, which depend on their sizes, shapes, and densities. This modification has been termed "gradient differential centrifugation" by Anderson (1955). The term "equilibrium zonal centrifugation" will be used for methods in which centrifugation is continued until most of the particles approach an isopycnic position. The separation is based mainly on the densities of the particles, even though not all are at true equilibrium at isopycnic positions. Long before this, small differences in densities will be magnified into large differences in sedimentation rates.

The purpose of the density gradient is to prevent convection and to keep the virus and other particles localized in zones. The efficiency of the method for separations follows directly from the differential migration of particles localized in zones. Visible virus zones are obtained if the virus is concentrated and pure enough (Fig. 1). Samples are readily

† Those gradients in which the property being considered increases with depth will be called positive.

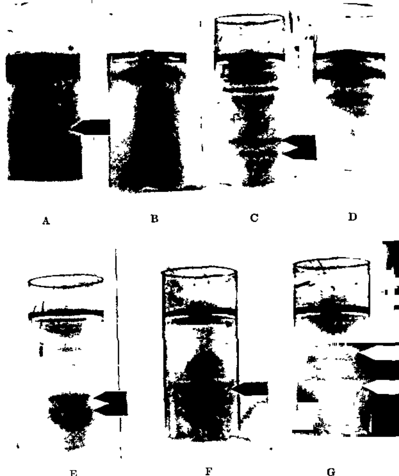


FIG 1 Gradient columns after centrifugation Tube A has an untreated extract from barley plants infected with barley stripe mosaic virus Tube B has an untreated extract from a healthy plant Tubes C and D have extracts from diseased and healthy barley plants, respectively, heated 1 hr at 40°C before centrifugation Tube E has an extract from a diseased barley plant shaken 3 minutes with *n*-butanol The gradient columns for tubes C, D, and E have 0.1% Igepon T-73 Tube F has tobacco mosaic virus and tube G has tobacco mosaic virus plus brome mosaic virus These last two viruses were purified by one cycle of differential centrifugation before rate zonal centrifugation All gradient columns were prepared from 4, 7, 7, and 7 ml of 0.01 *M* neutral phosphate buffer containing 100, 200, 300, and 400 mg sucrose per ml, respectively Centrifuged 1¾–2¾ hr at 23,000 rpm at 5°C in the SW25.1 rotor of the Spinco Model L centrifuge Arrows mark the location of virus zones Double zones are probably due to aggregation

removed for infectivity assays and electron microscopy, and a direct measure may be obtained of the sedimentation rate of the infectious particles and of the particles observed in the electron microscope. It is theoretically possible to calculate both the sedimentation constant and density of the solvated virus from the sedimentation rate of the virus at different depths. The density of the solvated virus may also be equated to the density of the medium at the position of the virus after isopycnic gradient centrifugation. The sedimentation coefficient and density are useful constants of themselves for characterizing viruses and, with further information and assumptions, may be used to calculate the weight and size of viruses. As with any purification procedure, density gradient centrifugation may be used to analyze virus preparations for purity and as a preliminary step in virus assay procedures based on weight or optical density.

The centrifugation of materials in a density gradient column to obtain a separation based on densities originated with E. N. Harvey (1931) and E. B. Harvey (1932) for *in situ* stratification of components of sea urchin eggs. Linderstrøm-Lang (1937) used density gradient columns of organic liquids to measure densities of water droplets. Behrens (1938, 1939), apparently independently of Linderstrøm-Lang, developed the use of density gradient columns for determination of the densities of dry cell nuclei and powdered "protoplasma" and for separation of these components. Centrifugation of density gradient columns was frequently used thereafter for determination of densities and separation of particles according to densities. Density gradient centrifugation for a separation of particles according to their sedimentation rates was reported by Brakke (1951, 1953) for purification of plant viruses. The essential difference from earlier techniques was use of a gradient column in which the particles being separated were soluble. It does not matter whether particles are aggregated if they are to be centrifuged until they have found a level with a density equal to their own, but it is essential that they be dispersed if they are to be separated from others on the basis of their sedimentation rates.

Steere (1959) has written a comprehensive review on purification of plant viruses, and Anderson (1956) has considered in detail the merits of differential centrifugation and density gradient centrifugation. These reviews should be consulted for information on many pertinent points which will not be considered here. The terms "virus" and "purification" will be used in the same sense and with the same reservations in mind as did Steere (1959). For the sake of conciseness, sedimentation of a virus in a column with a sucrose concentration gradient will be consid-

ered in much of the following discussion. Most of the comments would hold for other systems.

II Theory

A Introduction

The following symbols will be used:

- A_i: Weight in grams of component i in a zone
- a_i: Activity of component i
- c_i: Concentration of component i in grams per cm^3
- f_i: Frictional coefficient for sedimentation of component i
- g: Relative centrifugal force in gravity units
- λ: Thickness of a zone
- M_i: Molecular weight of component i
- P: Pressure
- R: Gas constant per mole
- r: Radius of gradient column in centimeters
- S_i: Sedimentation coefficient of component i
- s: Svedberg unit, equals 10^{-13} sec
- t: Time in seconds
- T: Temperature, degrees Kelvin
- W_i: Effective weight for sedimentation per particle of component i
- V_i: Partial specific volume of component i
- x: Distance from center of rotation in centimeters
- η: Viscosity
- ρ: Density of a solution in grams per cm^3
- ϕ_i: Effective density for sedimentation in solution of component i
- ω: *Angular velocity in radians per second*

The sedimentation coefficient of a particle is proportional to the weight of the particle in the suspending medium Most particles will be solvated in solution, that is, they will adsorb some of the components of the suspending medium It is the size and density of the solvated *particle that determine the sedimentation coefficient* For this purpose, those components of the medium adsorbed strongly enough to sediment with the particle must be considered as part of the solvated particle. The molecular weight and partial specific volume of an anhydrous component can be used in formulas for the sedimentation coefficient only if it can be assumed that the adsorbed part of the suspending medium has the same density as the bulk of the medium All available evidence indicates that this assumption does not hold for viruses dissolved in concentrated solutions of sucrose, proteins, inorganic salts, etc. In formulas in this review, the effective density, ϕ_i, of the solvated virus will be used instead of the partial specific volume of the solvated virus, because the former can be directly measured by sedimentation experiments, whereas the latter cannot be directly measured. The weight of the individual

solvated virus particle, W_i, will be used instead of molecular weight to avoid the implication that a solvated virus particle is a single molecule.

Symbols with prime marks (S'_i, ρ', η', ϕ', W'_i) will be used for values in sucrose solutions, or other solutions, under experimental conditions Symbols without prime marks will refer to values under standard conditions, i.e, water at 20°C Thus, S_i would be the sedimentation constant

B Role of the Density Gradient; Quantity of Virus in a Zone

The density gradient stabilizes the virus zone by preventing convection, but there is a theoretical upper limit to the amount of virus that may be contained in a stable zone in a density gradient column. The virus will have a negative concentration gradient in the lower part of its zone A density gradient will result from the negative virus concentration gradient and the positive sucrose concentration gradient. If there is to be no convection, the density gradient must be zero or positive. This imposes a limit on the magnitude of the virus concentration gradient in a stable zone in a given sucrose-concentration gradient.

The maximum amount of solvated virus in a stable zone may be calculated from Eq (1) derived by Svensson *et al.* (1957) and based on the assumption that the negative concentration gradient of the virus may increase until the density gradient approaches zero.

$$A'_i = \frac{\pi r^2 h^2 \left[\frac{d\rho'_1}{dx} - \left(\frac{d\rho}{dx}\right)_s\right]}{2\left(1 - \frac{\rho'_{1s}}{\phi'_i}\right)} \tag{1}$$

In this equation ρ'_1 is the density of the sucrose solution, and ρ'_{1s} is the value at the bottom of the zone. The term $(d\rho/dx)_s$ is a correction factor for the minimum over-all density gradient necessary for stabilization. This will have a small positive value for zone electrophoresis but should approach zero for centrifugation.

Equation (1) shows that the capacity of a zone should be directly proportional to the square of the zone thickness and to the density gradient, and inversely proportional to $(1 - \rho'_{1s}/\phi'_i)$, which is the weight of solvated virus of mass 1 gm. when suspended in a medium of density ρ'_{1s}. If a given density increment is available for a zone, the amount of virus in the zone would be directly proportional to the zone thickness. The total density range for a column is limited because of practical reasons, and, since resolution is determined by the ratio of zone thickness to column length, long columns may be used to process larger quantities than is possible in short columns with the same resolution

Capacity may be increased this way in zone electrophoresis, but not in zonal centrifugation where column length is limited

It was assumed in the derivation of Eq (1) that the sucrose concentration gradient was constant throughout the virus zone Actually, when virus sediments into a region, the solution would expand because of the volume occupied by the virus, and the sucrose concentration gradient would decrease This effect would be small with the virus concentrations used in practice

Svensson *et al* (1957) did not state the maximum amount of protein that had actually been contained in a stable zone Brakke (1955) could not obtain a stable zone containing 24 6 mg of hemoglobin with a thickness less than 1 3 cm. in a column of 16-mm diameter with a density gradient of 0 02 gm /cm^4 This is only about one-sixth the amount calculated by Eq (1) With smaller quantities of hemoglobin, the capacity of the zones was about one-third that predicted by Eq. (1) These zones were initially stabilized by stirring to prevent continued droplet sedimentation (see p 200) Part of the discrepancy between the observed and the theoretical capacity of the zones may have been due to this method of stabilization

In density gradient centrifugation the virus concentration gradient is automatically adjusted early in the centrifugation to give a stable zone (see p 200) As the amount of virus floated on a gradient column is increased, the thickness of the zone obtained after centrifugation is increased At higher virus concentrations the thickness of the final zone depends on the quantity of virus and not on the thickness of the original layer of virus solution (Brakke, 1953) In the latter case, the thickness of the zone appears to be determined and limited by conditions necessary for its stability These conditions apparently are not the same as those embodied in Eq (1), because it has not been possible to contain more than a small fraction of the amount of virus predicted by Eq (1) in a stable zone According to equation (1), with a 1-in diameter tube, a gradient of 50 mg sucrose/cm^4, $\rho'_{1x} = 1\ 10$, and $\phi' = 1.25$, a zone 1 cm thick should contain 420 mg of virus, a zone 5 mm thick should contain 105 mg of virus, and a zone 2 mm thick should contain 17 mg of virus In practice the capacity of zones is only a fraction of this Ten milligrams of either tobacco mosaic or southern bean mosaic viruses gives a zone about 2 cm thick under these conditions (Brakke, unpublished) The boundaries of the zones with high concentrations of virus are not sharp, and no accurate measurements of zone thickness have been made Nevertheless, the capacity of the gradient column for supporting virus in a zone clearly falls far short of theoretical In practice, 1-in diameter gradient columns of 50 mg sucrose/cm^4 are most useful for

solvated virus particle, W_i, will be used instead of molecular weight to avoid the implication that a solvated virus particle is a single molecule.

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droplets would soon reach an isopycnic position and, being small, would disperse by diffusion. Repeated formation and sedimentation of such small droplets followed by their dispersion would lead to a more diffuse boundary for the virus zone and a small, negative virus concentration gradient.

It has been observed (Brakke, 1953) that virus zones become thicker when they first sediment into the sucrose gradient column They appear to be stable as soon as they have been centrifuged The above explanation satisfactorily explains all observations except the very low capacity of the zones The fact that zones with a given quantity of virus are observed to be thicker than expected must be related to the events that occur when the virus first enters the gradient column, as well as to the conditions necessary for a stable zone within the column Since the capacity of a zone falls far short of the theoretical amount as expressed in Eq (1), the above hypothesis of events when the virus first sediments into the gradient column must be considered incomplete.

D Estimation of Sedimentation Constants and Densities

Viruses appear to sediment through gradient columns at the rate one would expect from the theory developed for sedimentation by Svedberg and Pedersen (1940) and others The sedimentation coefficient, S', is equal to the sedimentation rate reduced to unit centrifugal force and unit time

$$S' = \frac{1}{\omega^2 x} \frac{dx}{dt} \tag{2}$$

The sedimentation coefficient may be expressed as Svedberg units, defined as cm $\times 10^{-13}$ per sec per dyne per gram or 10^{-13} sec The sedimentation coefficient of a solvated particle, ,, theoretically is a function of its size, shape, and density as expressed by Eq (3)

$$S'_s = \frac{W'_s(\phi'_s - \rho')}{\phi' f'_s \eta'} \tag{3}$$

The sedimentation coefficient under standard conditions will be called the sedimentation constant It is given by

$$S_s = \frac{W_s(\phi_s - \rho)}{\phi f_s \eta} \tag{4}$$

Combining Eqs. (3) and (4) gives the relation between the sedimentation coefficient and the sedimentation constant.

$$S_s = S'_s \frac{W_s f'_s \phi' \eta' (\phi_s - \rho)}{W'_s f_s \phi \eta (\phi'_s - \rho')} \tag{5}$$

0 01 to 1 0 mg. of virus. If the virus is comparatively pure, even 0 01 mg gives an easily visible zone. Lesser quantities may be used if some criterion other than visibility is employed for locating the virus.

Equation (1) gives an upper limit for the capacity of a stable zone in a density gradient column based on the proposition that the density gradient must be positive at all places. Simplifying approximations made in the mathematical derivation and solvation effects could account for small discrepancies between theory and practice but not for the marked discrepancy observed. It must be concluded that some unknown phenomenon is limiting the capacity of the virus zones in a gradient column before the density gradient approaches zero. Local variations in concentrations of particles, as suggested by Anderson (1956), might be the explanation, but no mathematical exploration has been made to see whether expected statistical variations would explain the observed effects

C. Prevention of Droplet Sedimentation

When a layer of virus solution is floated on top of a gradient column, the virus concentration gradient at the boundary between virus and sucrose is large and negative, whereas the sucrose concentration gradient is large and positive The density gradient must be positive, or the virus solution would not float If the column is not centrifuged, the sucrose concentration gradient will decrease much more rapidly than the virus concentration gradient decreases, because sucrose diffuses faster than virus. As the sucrose concentration gradient decreases, a point is reached where it is not great enough to counteract the negative virus concentration gradient, and convection begins. Small droplets may be observed to settle In density gradient zone electrophoresis several methods may be used to prevent this droplet sedimentation and obtain stable zones Brakke (1955) stabilized protein and virus zones by gently stirring the droplets to disperse them. Svensson *et al.* (1957) discuss several theoretical methods of stabilizing such zones and in practice has used upward migration from a zone added at a density shelf.

If a gradient column is centrifuged soon after the virus solution is floated on it, stable virus zones are obtained By some mechanism the virus concentration gradient at the bottom of the zone has been decreased enough so that the virus zone is stable. One may suggest the hypothesis that a negative density gradient would be formed as the bottom of the virus zone with its large, negative concentration gradient migrates past the level of the original surface of the sucrose solution The droplets that would start to sediment in the narrow region of the negative density gradient would be small because of the high centrifugal force. These

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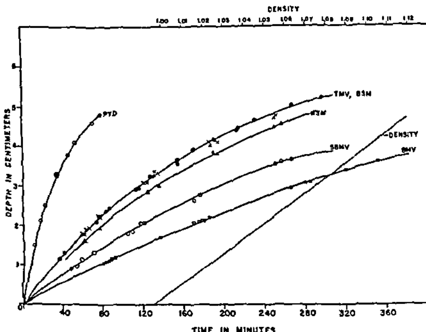


FIG 2 Depth of viruses after centrifugation for various periods through a gradient column prepared from 3, 7, 7, 7, and 4 ml of solutions containing 0, 100, 200, 300, and 400 mg sucrose/ml, respectively Temperature after centrifugation was 5-6°C Centrifuged at 24,000 r p.m in the SW25 1 rotor of the Spinco Model L centrifuge The density curve is an average from several measurements PYDV = potato yellow dwarf virus, TMV(●) = tobacco mosaic virus, BSMV (X) = barley stripe mosaic virus, WSMV = wheat streak mosaic virus, SBMV = southern bean mosaic virus, BMV = brome mosaic virus (After Brakke, 1958a)

is equal to the slope of the curve for that virus at the specified depth It is easier to measure the depth after a period of centrifugation than it is to measure the sedimentation rate at a given place in the gradient column Therefore, combinations of Eqs (2) and (3) are sometimes used after integration to calculate the sedimentation constant or some other parameter of particle size

If Eqs (2) and (3) are combined, one gets

$$t_2 - t_1 = \int_{x=1}^{x=2} \frac{\phi' f'_{s,t}\eta' \, dx}{W'_s \omega^2 x(\phi'_s - \rho')} \tag{7}$$

Thomson and Mikuta (1954) and Thomson and Moss (1955) expressed the viscosity as a polynomial in third power of x for a gradient column

The weight and size of a particle can be calculated from the sedimentation coefficient with Eq (3) if the frictional coefficient and density of the particle are known To obtain these requires additional assumptions or additional measurements or both Since further errors may be introduced with these measurements and assumptions, it is preferable to report sedimentation rate data as the sedimentation constant rather than as particle size The sedimentation constant has a characteristic value for each virus and is as useful as any other measure of size for describing viruses.

It should be possible from measurements of sedimentation rates of a virus in a density gradient column to calculate both the sedimentation constant and the effective density of the solvated virus Equation (5) may be rearranged to give

$$\frac{\eta'}{\eta} S'_i = S_i \frac{W'_i f_{i,s}}{W_i f'_{i,s}} - \frac{S_i W'_i f_{i,s}(\rho' - \rho)}{W_i f'_{i,s}(\phi_i - \rho)} \tag{6}$$

if it is assumed that $\phi'_i = \phi_i$ It is obvious that both S_i and ϕ_i may be calculated from measurements of S'_i at different values of ρ' if $W'_i f_{i,s}/W_i f'_{i,s}$ is equal to 1 A plot of $\eta' S'_i/\eta$ vs. $\rho' - \rho$ should give a straight line if $W'_i f_{i,s}/W_i f'_{i,s}$ and ϕ'_i are constant and not dependent on ρ'. If such a plot does not give a straight line, at least one of these properties must be changing as the virus sediments through the gradient column.

A gradient column will usually have viscosity and osmotic pressure gradients as well as a density gradient. One of the components of the gradient column may be preferentially adsorbed by the virus, and the extent of this preferential adsorption may change with depth because of the concentration gradients If water is preferentially adsorbed by the virus, the hydration of the virus might decrease as the concentration of sucrose increases Since any of the components of the gradient column could be preferentially adsorbed by the virus, the term "solvation" will be used instead of "hydration" If there is a volume change involved in the solvation reaction, the extent of solvation may change as the virus sediments down the tube because of the increasing pressure. If the solvation of the virus is not constant, W'_i, $f'_{i,s}$, and ϕ'_i will be functions of depth, and their determination will be difficult or impossible. It is important to note that, in most treatments, these variables have been assumed to be constant

The depth of a virus zone in a gradient column after a period of centrifugation may be measured after the centrifuge is stopped. A plot of depth vs. time of centrifugation gives the curves shown in Fig 2 for some plant viruses. The sedimentation rate of a virus at any depth

took this ratio to be approximately equal to the ratios of times required for the viruses to sediment through a given depth increment, these times being determined graphically from the curves in Fig 2 The error in this approximation depends mainly on the difference between the effective densities of the two solvated viruses The standard error of estimate of ratios of sedimentation constants by this method varied from 1 to 3 5% The ratio of the sedimentation constant of southern bean mosaic virus to that of tobacco mosaic virus was 0 59 by this method Values of sedimentation constants of these two viruses from the literature gave a ratio of 0 58–0 62. The range is due mainly to the fact that different values for the sedimentation constant of tobacco mosaic virus have been reported The ratio of the sedimentation constants of barley stripe mosaic and tobacco mosaic viruses was 1 01, of wheat streak mosaic and tobacco mosaic virus, 0 86; of brome mosaic and tobacco mosaic viruses, 0 41; and of potato yellow dwarf and tobacco mosaic viruses, 4 4–4 8 The effective densities calculated by this method were 1.28 for wheat streak mosaic virus, 1 24 for barley stripe mosaic virus, 1 36 for brome mosaic virus, 1 17 for potato yellow dwarf virus, and 1.26 for southern bean mosaic virus Lauffer *et al* (1952) reported values of 1.26 and 1.24 for southern bean mosaic virus, one value being obtained by interpolation and the other by extrapolation

E. Determination of Densities by Isopycnic Gradient Centrifugation

In these techniques, centrifugation is continued until equilibrium is reached with the virus at an isopycnic position in the gradient column This is a static method of determining densities in contrast to the above methods where densities of particles are determined from measurements of sedimentation rates in media of different densities The principle of isopycnic gradient centrifugation was introduced by Linderstrøm-Lang (1937) and Behrens (1938, 1939) It is easier to tell when equilibrium has been reached if the virus is present throughout the tube before centrifugation so that it both floats and sediments toward the isopycnic position. Before equilibrium, the virus accumulates in two zones, one just above and one just below the isopycnic position (Kahler and Lloyd, 1951) The accumulation in the top zone occurs first, because of the lower viscosity in the upper parts of the column Care must be taken not to confuse this first accumulation of virus above the isopycnic position for the zone that will eventually form at the true isopycnic position.

Perhaps because of the long centrifugation required, this method has seldom been used to determine effective densities of viruses in sucrose solutions Kahler *et al* (1954) found the density of Rous sarcoma virus

in which the density was a linear function of x and integrated Eq (7). They assumed that W'_i, $f'_{i,s}$, and ϕ'_i were independent of x and that all particles started to sediment at the meniscus. The resulting equation is complex and requires the evaluation of the four arbitrary constants used in expressing viscosity and density as functions of distance from the center of rotation These constants will be functions of the temperature as well as of the sucrose concentration gradient. The diameter of a particle can be calculated from its depth in a gradient column by the formula of Thomson and Mikuta if one assumes a value for ϕ'_i, and assumes that all particles are spherical.

Thomson and Klipfel (1958) have presented a simpler integrated form of Eq (7) for polyvinylpyrrolidone-water concentration gradients The viscosity could be represented by a quadratic equation in x for this system

Combination of Eqs. (5) and (2) with the assumption that

$$W_i f'_{i,s}/W'_i f_{i,s}$$

equals 1 and that $\phi'_i = \phi_i$ leads to

$$S_i = \frac{\phi_i - \rho}{\omega^2 \eta (t_2 - t_1)} \int_{x=1}^{x=2} \frac{\eta' \, dx}{x(\phi_i - \rho')} \tag{8}$$

By numerical integration of Eq (8) and using data from density gradient centrifugation, Strohmaier and Mussgay (1959) obtained a value of 104s for the sedimentation constant of hemocyanin from *Helix pomatia* The analytical ultracentrifuge yielded a value of 105s Having thus demonstrated the reliability of the density gradient method, Strohmaier and Mussgay determined a mean value of 37s for the sedimentation constant of the infectious unit of ribonucleic acid preparations from foot-and-mouth disease virus

Brakke (1958a) determined the ratio of sedimentation coefficients of two viruses to avoid the necessity of determining the viscosity in the gradient column From Eqs (4) and (3) for two viruses, designated by subscripts m and n, Eq (9) may be derived

$$\frac{(\phi_n - \rho')S'_m}{(\phi_n - \rho)S'_n} = \frac{S_m}{S_n} - \frac{S_m(\rho' - \rho)}{S_n(\phi_m - \rho)} \tag{9}$$

It is assumed in the derivation that $W'_m W_n / W_m W'_n$ and $f_{m,s} f'_{n,s} / f'_{m,s} f_{n,s}$ are both equal to 1 and that $\phi'_m = \phi_m$ and $\phi'_n = \phi_n$. A plot of the left-hand member of Eq. (9) versus $\rho' - \rho$ should give a straight line with an intercept of S_m/S_n and a slope of $S_m/S_n(\phi_m - \rho)$ If the sedimentation coefficient and effective density of one virus are known, it is possible to calculate the sedimentation coefficient and effective density of the second virus. The ratio of S'_m/S'_n must be determined experimentally Brakke

tration curve in the method of Meselson *et al* (1957) As a result, the molecular weight of the polymer would be underestimated Yeandle (1959) has considered the error in the molecular weights calculated by the method of Meselson *et al* due to neglect of charge effects He concluded that this error should be less than 15% with deoxyribonucleic acid

Siegel and Hudson (1959) reported the density of U1 strain of tobacco mosaic virus to be 1 306, 1 316, 1 307, 1 325, and 1 325 gm /cm³ when measured by the method of Meselson *et al* (1957) in solutions of KBr, NaBr, RbBr, RbCl, and CsCl, respectively The U2 strain consistently gave a value of 0 003 gm /cm³ lower than did the U1 strain These values may be compared with the value of 1.27 gm /cm³ obtained by Schachman and Lauffer (1949) by a flotation method for the density of tobacco mosaic virus in sucrose solutions

Most investigators determining densities by isopycnic gradient centrifugation in concentrated salt solutions have used the analytical ultracentrifuge However, Kozinski and Szybalski (1959) measured the density of normal ϕX-174 bacteriophage, and of the same bacteriophage containing 5-bromouracil, by centrifugation in a cesium chloride solution in a swinging bucket rotor The density and bacteriophage titer of samples of solution removed from the tube after centrifugation were measured, and the effective density of the bacteriophage determined from these data

As with all values of density determined by flotation methods, the densities determined by this method are functions of the experimental conditions The density determined is that of a solvated particle whose composition is unknown The solvation will depend on the composition of the supporting medium The effect of the increase in pressure during centrifugation on density measurements will be discussed in Section G

F The Wall Effect

It is generally assumed that those particles that congregate near the walls of a centrifuge tube, as they sediment straight away from the center of rotation, are subsequently carried to the bottom of the tube by the flow of the concentrated solution which they form This phenomenon is accentuated in rotors having tubes held at a fixed angle, and is undoubtedly responsible for the poor results obtained when angle rotors are used for density gradient centrifugation Its occurrence is not as well established for centrifugation in rotors with swinging buckets, with which the rest of this section is concerned Anderson (1955) used sector-shaped centrifuge tubes to eliminate the possibility of this wall sedimentation during density gradient centrifugation Thomson and

to be 1.15 in aqueous sucrose solutions and 1.174 in solutions of sucrose in 65% aqueous D_2O.

Meselson *et al.* (1957) measured the densities of nucleic acids by isopycnic gradient centrifugation in a concentration gradient of cesium chloride. Contrary to previous practice, the concentration gradient of cesium chloride was not preformed, but arose from partial sedimentation of the cesium chloride during centrifugation. The concentration gradient at equilibrium of a component at depth x during centrifugation is given by

$$\frac{dc_i}{dx} = \frac{dc_i}{da_i} \frac{a_i M_i (1 - V'_i \rho'_x) \omega^2 x}{RT} \tag{10}$$

where ρ'_x is the density at depth x. The concentration gradient, and the density gradient, will depend on the centrifugal force, and on the concentration, molecular weight, and partial specific volume of component i.

The order of magnitude of sucrose concentration gradients at equilibrium may be obtained by assuming c_s equal to a_s, and the partial specific volume of sucrose equal to 0.60. Substitution in Eq. (10) gives

$$\frac{dc_s}{dx} = 5.8 \times 10^{-6} c_s g - 3.3 \times 10^{-6} c_s^2 g \tag{11}$$

With a relative centrifugal force of 100,000 times gravity, the sucrose concentration gradient would be 54 mg./cm.^{4} if the sucrose concentration were 100 mg./cm.^{3}, and 235 mg./cm.^{4} if the sucrose concentration were 600 mg./cm.^{3} These are gradients of the same order of magnitude as the preformed sucrose concentration gradients usually used.

Inorganic salts have the advantage over sucrose that their solutions have a lower viscosity for a given density, and that higher densities may be obtained than with sucrose solutions. With 8.9 M cesium chloride, Meselson *et al.* (1957) measured nucleic acid densities as high as 1.80. These authors derived formulas for calculation of the molecular weight of the nucleic acids from the width of the nucleic acid zone at equilibrium. They obtained a value of 1.70 for the density of deoxyribonucleic acid from T_2 bacteriophage and 14×10^6 for the molecular weight. The suspending medium was 7.7 M cesium chloride, and the density gradient was 0.046 gm./cm.^{4} at a speed of 27,690 r.p.m. in the Spinco Model E ultracentrifuge.* An accuracy of 0.001 gm./cm.^{3} is claimed for density measurements under these conditions.

Baldwin (1959) has shown that polymers heterogenous in density, but with a gaussian distribution of densities, should give a gaussian concen-

* Mention of specific equipment or products does not constitute endorsement by the U. S. Government.

increase in density at the bottom of the tube would be about 0.018 gm/cm^3. In the SW39 rotor of the same centrifuge, the pressure at the bottom of a 5-cm column of water would be about 640 atmospheres at 40,000 rpm, giving an increase in density for water of about 0.025 gm/cm.^{3}.

Cheng and Schachman (1955) utilized a density gradient produced by compression of D_2O–H_2O during centrifugation to float polystyrene latex at the bottom of the centrifuge cell while it sedimented at the top. From the results they calculated the compressibility of the latex. They pointed out that if they had used the equilibrium position of the polystyrene latex to calculate the density of the latex, the results would have depended on the speed of rotation of the centrifuge and on the depth in the cell of the zone at equilibrium.

The extent of solvation will change with a change in pressure if the solvation of the virus is accompanied by a volume change. It has been postulated that water of hydration is essentially frozen water (e.g. see Klotz, 1958) and has a greater molar volume than does liquid water. If this be the case, the extent of hydration should decrease with increasing pressure. A decrease in hydration could account for a greater change in effective density of a virus than that due to the difference in compressibility of the virus and the suspending medium.

These compression effects are important chiefly in the measurement of densities of viruses. The effect on measurement of the sedimentation rates and calculation of sedimentation constants will be small, unless the density of the solution is almost the same as that of the virus (Schachman and Lauffer, 1950, Fujita, 1956). In general, density of a virus may be determined in three ways by centrifugation—by extrapolation from sedimentation rates measured in the upper part of the tube versus solvent density, by extrapolation from flotation rates measured in the lower part of the tube, and by determination of the isopycnic position of the virus zone in a gradient column. Even with the same suspending medium, the results may be expected to vary with the experimental procedure.

H. Resolution

The high resolution of rate zonal centrifugation as compared with differential centrifugation (cycles of alternate low- and high-speed centrifugation) is a direct result of differential migration of zones in the former technique. Figure 1, tube G, shows the complete separation of brome mosaic and tobacco mosaic viruses by rate zonal centrifugation. Samples from the brome mosaic virus zone in tubes such as this have no detectable tobacco mosaic virus. Samples from the tobacco mosaic virus zone have

Mikuta (1954) corrected for wall sedimentation. Unpublished experiments from the author's laboratory have shown that this phenomenon does not always occur in density gradient centrifugation in swinging bucket rotors. Southern bean mosaic virus was purified by a preliminary density gradient centrifugation to remove aggregated particles Samples from the zone of this first gradient column were removed, diluted, and floated on a second gradient column The columns had a gradient of 50 mg. sucrose/cm.[4], and 0 2 to 1.4 mg of virus per column After centrifugation, the resuspended pellet from this second gradient column had less than 1% as much virus (by infectivity assay) as did samples from the virus zone. The pellet should have had one-third as much virus as did the zone if the virus that theoretically struck the wall had all sedimented to the pellet Wall sedimentation apparently did not occur under these conditions It may occur under other conditions However, it is plain that one cannot assume under any conditions that all the virus that theoretically should strike the wall will be found in the pellet

Hogeboom and Kuff (1954) reported that the concentration of protein in the plateau region during centrifugation in a cylindrical tube did not decrease as rapidly as would be expected if the wall effect were fully operative The tubes contained a slight sucrose concentration gradient to help prevent convection.

The reasons for the absence of wall effects under certain conditions in density gradient centrifugation are not known with certainty. Possibly the heavier solution formed near the wall by a congregation of particles flows a short distance down the tube and then back toward the center of the tube as it reaches a position where the supporting sucrose solution has an equal or slightly greater density.

G Compression Effects

The high pressure developed during centrifugation causes compression of both virus and suspending medium and may change the degree of solvation, if the solvation reaction is accompanied by volume changes The effects may be important in density measurements.

The pressure, P_2, in a centrifuge tube at depth x_2 may be calculated from the formula

$$P_2 - P_1 = \omega^2 \int_1^{x_2} \rho x \, dx \qquad (12)$$

where P_1 is the pressure at the meniscus, x_1 At the bottom of a 7-cm column of water in the SW25 1 rotor of the Spinco Model L centrifuge, the pressure would be about 450 atmospheres at 25,000 r.p m. The

increase in density at the bottom of the tube would be about 0 018 gm/cm^3. In the SW39 rotor of the same centrifuge, the pressure at the bottom of a 5-cm. column of water would be about 640 atmospheres at 40,000 r p m, giving an increase in density for water of about 0 025 gm/cm.^{3}.

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about 0 1% the original concentration of brome mosaic virus (unpublished data) To obtain a similar degree of separation by differential centrifugation would require at least 7 or 8 cycles since brome mosaic sediments 41% as fast as tobacco mosaic virus at infinite dilution

The resolution of any separation method based on differential migration of zones depends on the thickness of the zones and the distance of migration The thickness of virus zones in a gradient column depends on the amount of virus, and it follows that resolution will become poorer as the concentration of virus is increased The distance available for migration is limited by the length of the centrifuge tube. As the virus migrates into a more concentrated sucrose solution, its sedimentation rate decreases, as shown in Fig 2. The increase in density of the sucrose solution is partly responsible for this decrease in sedimentation rate, but the increase in viscosity is more important. Obviously, the resolution would be improved if a faster sedimentation rate were maintained throughout the column This could be accomplished by using a lesser gradient, but in this case the width of the zones would increase, and a net improvement in resolution might or might not be achieved. The viscosity of a concentrated sucrose solution decreases more rapidly with increasing temperature than does the viscosity of a dilute solution Therefore, the sedimentation rate of a virus should not decrease as rapidly with increasing sucrose concentration at higher temperatures as it does at lower temperatures. Resolution in rate zonal centrifugation should improve with an increase in temperature.

It is apparent from inspection of Fig 2 that complete separation of two viruses could be obtained under conditions of that experiment if one virus sedimented 75% as fast as the other

In theory, it should be possible to increase the resolution in isopycnic gradient centrifugation almost without limit by decreasing the density gradient. However, there is a limit to the amount the density gradient can be decreased The sucrose concentration gradient will have an equilibrium value dependent on the speed of centrifugation The slower the centrifugation, the smaller the density gradient, the higher the resolution, and the longer it will take the virus to reach equilibrium Thus the time required for the centrifugation may limit the resolution

It should be possible to increase the resolution in equilibrium zonal centrifugation by using a gradual gradient In practice, the slow sedimentation rates make it desirable to use a short column of a density range selected for the problem at hand. The steepness of the gradient will depend on whether high resolution or high concentration of the virus in the final zone is more important

III. PROCEDURES

A. *Materials for Preparing Density Gradient Columns*

An ideal material for preparing density gradient columns should be chemically inert, readily available, and of a high molecular weight, and should give solutions of high density and low viscosity. Sucrose is the most commonly used substance for gradients for plant viruses. Glycerine, because of its volatility, is convenient when the virus is to be used for electron microscopy. A disadvantage of glycerine, however, is the fact that its solutions have a higher viscosity and osmotic pressure than do sucrose solutions of the same densities. Both sucrose and glycerine are usually without detrimental effects on viruses and may even have a protective action.

Deuterium oxide, alone or in combination with sucrose, has been used in gradient columns by Kahler *et al.* (1954) and by Strohmaier and Mussgay (1959). The main advantage of deuterium oxide is its chemical similarity to water. Practical disadvantages are the high cost and the relatively low densities obtainable.

Holter *et al.* (1953) reported the use of gradient columns of sucrose and "Diodon" (di-ethanol-amine salt of 3,5-diiodo-4-pyridone-*N*-acetic acid) for centrifugal separation of cytoplasmic components. Diodon is commercially available as a 70% aqueous solution (density 1.36) for X-ray photography. Diodon has a higher molecular weight than does sucrose, but otherwise its superiority to sucrose is not obvious in absence of viscosity data on solutions. The density of a 70% sucrose solution (1.35) is almost the same as that of 70% Diodon.

Recently Holter and Møller (1958) have reported the use of "Ficoll" for density gradient columns. This is a commercially available chemical of unspecified composition. Since the molecular weight is about 50,000, it should have a small osmotic pressure effect. The density of a Ficoll solution is not quite as much as that of a sucrose solution of the same concentration. A solution of 2.19 gm. of Ficoll in 2 gm. of water has a density of 1.21 gm./cm.^{3}, whereas a similar concentration of sucrose has a density of 1.24 gm./cm.^{3} No information has been published on viscosity of Ficoll solutions.

Thomson and Klipfel (1958) have used polyvinylpyrrolidone for density gradient columns. Its solutions have a much lower osmotic pressure but much greater viscosity than do sucrose solutions of the same density.

Aqueous solutions of substances lighter than water could also be used for density gradient columns. Isopycnic separations of viruses could

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A gradient-mixing device produces a gradient by mixing effluents from two or more chambers containing solutions of different concentrations The first such device was described by Behrens (1938) Mercury from a common reservoir flowed into the bottoms of two parallel reservoirs, one containing a light and the other a heavy solution Effluents of the two reservoirs flowed into and through a common capillary and dripped onto a flat cork floating on the gradient column The cross-sectional area of the reservoir with the light solution increased with height, and the cross-sectional area of the other reservoir decreased with height.

Equations describing gradients produced by reservoirs in series, i e, with a second reservoir emptying into the first which is continuously stirred, have been presented by Drake (1955), Svensson (1958), and Lakshmanan and Lieberman (1954). The last authors describe a device in which the contents of an upper reservoir flow into a lower reservoir containing a magnetic stirrer. The rates of flow into and out of the lower reservoir are the same, and its effluent has a concentration gradient. Similar devices have been described repeatedly since Bock and Lang (1954) described a device, similar in principle to that of Behrens, for producing gradients of any desired specification Effluents flowing by gravity from two parallel reservoirs were mixed in a capillary or a very small mixing chamber to produce a gradient The reservoirs may be constructed of plastic in a variety of shapes to give any desired gradient.

Anderson (1955, 1956) described a gradient-producing engine in which the effluents from two syringes were mixed in a small mixing chamber with a magnetic stirrer. A more reliable rate of flow was obtained than with gravity flow from reservoirs described by Bock and Lang The effluent from the mixing chamber was led to the bottom of the centrifuge tube, the lightest solution being introduced first. The plungers of the syringes were driven at differential rates by a motor to give reproducible flow rates It required 90 minutes to produce a gradient column of 60–70 ml. with this engine Sinclair *et al.* (1957) described a similar device. More recently, Albright and Anderson (1958) described a centrifuge rotor in which the density gradient column can be formed during centrifugation. This apparatus allows very rapid separation of large cellular components, but the rotor does not have sufficient speed for virus separations

The most commonly used gradient column for rate zonal separations has 0–40% sucrose The most suitable range for the density gradient is 0 01–0 04 gm /cm.⁴ corresponding to a sucrose concentration gradient of 25–100 mg /cm.⁴. The sucrose is dissolved in a suitable buffer, usually one in which the virus is stable and adequately dispersed. Reducing

not be carried out in such columns, but sedimentation rate separations could be. Charlwood and Gordon (1958) have used ethanol-water density gradient columns for zone electrophoresis. The low viscosity of ethanol-water columns would make rapid separations possible. The tendency of ethanol to precipitate and denature some viruses would be a disadvantage.

The use of inorganic salts for isopycnic gradient centrifugation has been discussed on pp. 206–7

B Formation of Gradient Columns

Gradient columns may be formed with gradient-producing machines, or by layering different concentrations of aqueous sucrose one on the other, followed by aging during which diffusion produces a smooth gradient. The simplest procedure for layering solutions of different densities is to float one slowly on top of another with a pipette The pipette should have a large orifice so that the emerging liquid will have a low linear velocity. The allowable flow rate is increased if the tip of the pipette is bent into a U-shape and placed at the surface of the next heavier layer so that emerging liquid flows upward

Brakke (1953) used funnels with thin stems extending to the bottom of the centrifuge tubes for simultaneous preparation of several gradient columns The lightest solution was added first followed by heavier ones in order, each solution flowing under the previous one. With 1-in. diameter tubes, as used in the SW25 1 rotor of the Spinco Model L centrifuge, the constrictions (specified by Brakke) in the necks of the funnels are unnecessary, since small air bubbles from the funnel stems do not cause appreciable convection in these tubes.

The time required for a smooth gradient to be formed from a series of layered solutions depends on the width of each layer. Brakke (1953) found that one day was sufficient for a smooth gradient to be formed from layers initially 1.5 cm. thick Droplets of mixtures of bromobenzene-paraffin oil were floated in the column to determine densities at different depths. Ottesen and Weber (1955) and Weber (1956) reported that it took 10 hours to form a sufficiently smooth gradient from layers originally 6 mm. thick with increments of 7.5% sucrose between layers. To test this point, they prepared a gradient column in a square cuvette and placed this in the Tiselius electrophoresis apparatus to observe the refractive index changes Anderson (1955) decided on the basis of refractometric analyses that this method of producing gradients was unsatisfactory. His sucrose solution layers may have been wider to start with than were those used by Brakke or Weber and Ottesen.

column in 12 hours, and that the gradient in this part of the column did not change much during the next day and a half or during centrifugation The beads near the top and bottom of the column changed positions with aging of the column and during centrifugation more than did the beads in the central part of the column Experience with droplets of bromobenzene-paraffin oil mixtures in the author's laboratory has confirmed these results Since a sucrose concentration gradient will be present at equilibrium during centrifugation, a preformed gradient should be more stable during centrifugation than otherwise

C Preparation of Virus Sample and Centrifugation

The treatment of the virus sample will depend on the virus and on the purpose of the experiment For most purposes, a 5-mm. layer of virus solution floated on top of the gradient column is satisfactory The concentration of virus should be less than 0 5 mg per ml for best resolution in rate zonal centrifugation Ten times as much may be used for purification of larger quantities if a poorer resolution is acceptable The concentration of many plant viruses in crude extracts is high enough for use without further concentration Extracts of plants with tobacco mosaic may have too much virus for good resolution With most plant viruses, some of the normal plant components must be denatured (by freezing, heating, treatment with organic solvents, etc) and removed by low-speed centrifugation if a visible virus zone is to be obtained Calcium phosphate adsorption of normal components appears to be promising (Fulton, 1959) and, with barley stripe mosaic virus, separation of normal host components is facilitated by dispersion with a detergent (Brakke, 1959) The effect of some of these procedures with barley stripe mosaic virus is illustrated in Fig 1

Many of the normal components of plants appear to have a low density and may reach an isopycnic position after centrifugation in a 0–40% sucrose column The exact conditions of the preceding low-speed centrifugation of a crude extract from diseased plants often has a surprisingly large effect on the results obtained in rate zonal centrifugation Dilution of the crude extract, time and speed of centrifugation, and volume in the centrifuge tube are important

Gradient columns should be centrifuged immediately after the virus solution has been floated on them If they are allowed to stand, droplet sedimentation will develop (Brakke, 1953, called "streaming effect" by Anderson 1955, 1956), and the virus will soon be distributed throughout the column

It is, of course, necessary to handle gradient columns gently both before and after centrifugation to prevent unnecessary mixing Ander-

agents, divalent cations, or chelating agents, etc, may be added if desired. The gradient column usually employed in the author's laboratory is prepared by layering 4, 7, 7, and 7 ml. of 0 01 *M* neutral potassium phosphate buffer containing 100, 200, 300, and 400 mg. sucrose per ml, respectively, in a 1 in × 3 in. tube. Using funnels (Brakke, 1953), one person can prepare 10 gradient columns in 30 minutes. These columns can be used after standing at least one day, but not more than one week, at 0–2°C. Contamination with microorganisms is a problem if the columns stand a week or more.

Columns for equilibrium zonal centrifugation should have a maximum of about 600 mg of sucrose/ml. (density 1 23). Most plant viruses have densities greater than 1 23, but many normal components of plants have densities less than this. If the virus is added as a zone at the top of the column, it is not necessary to centrifuge until the virus and all impurities have reached isopycnic positions to obtain a separation based mainly on densities As particles approach isopycnic positions, small differences in densities will cause larger and larger differences in sedimentation rates. It would often require a prohibitively long time to centrifuge until all particles had reached isopycnic positions It usually is feasible to centrifuge until small differences in density have caused large enough differences in sedimentation rates so that the zones have separated

The gradient columns used in the author's laboratory for equilibrium zonal centrifugation are formed from 0 8 to 1.0-cm layers of buffer solutions containing 300, 400, 500, and 600 mg. sucrose per milliliter. Because of their high viscosities, these solutions are more quickly layered with a pipette than with funnels

A uniform gradient is produced in the center of the column by diffusion from layers of sucrose solution However, the gradient will be less near the top and the bottom of the column (Ottesen and Weber, 1955). This defect can be overcome by using a gradient-producing engine, by removing the top 0 5–1 0 cm of the gradient column just before use, or by using 5–10% sucrose for the lightest solution. If a virus solution with no sucrose is floated on a gradient column with a minimum of 10% sucrose, the gradient at the top of the column will be increased by diffusion and some convection while the virus solution is being floated and the centrifuge rotor loaded.

Ottesen and Weber (1955) give directions for preparing glass beads with air bubbles of different sizes for measuring densities in gradient columns. Brakke (1953, 1958a) has used droplets of mixtures of bromobenzene-paraffin oil for the same purpose Ottesen and Weber observed that a smooth, uniform gradient was formed in the central part of the

column in 12 hours, and that the gradient in this part of the column did not change much during the next day and a half or during centrifugation The beads near the top and bottom of the column changed positions with aging of the column and during centrifugation more than did the beads in the central part of the column Experience with droplets of bromobenzene-paraffin oil mixtures in the author's laboratory has confirmed these results. Since a sucrose concentration gradient will be present at equilibrium during centrifugation, a preformed gradient should be more stable during centrifugation than otherwise

C Preparation of Virus Sample and Centrifugation

The treatment of the virus sample will depend on the virus and on the purpose of the experiment For most purposes, a 5-mm layer of virus solution floated on top of the gradient column is satisfactory The concentration of virus should be less than 0 5 mg per ml for best resolution in rate zonal centrifugation Ten times as much may be used for purification of larger quantities if a poorer resolution is acceptable The concentration of many plant viruses in crude extracts is high enough for use without further concentration Extracts of plants with tobacco mosaic may have too much virus for good resolution With most plant viruses, some of the normal plant components must be denatured (by freezing, heating, treatment with organic solvents, etc) and removed by low-speed centrifugation if a visible virus zone is to be obtained Calcium phosphate adsorption of normal components appears to be promising (Fulton, 1959) and, with barley stripe mosaic virus, separation of normal host components is facilitated by dispersion with a detergent (Brakke, 1959) The effect of some of these procedures with barley stripe mosaic virus is illustrated in Fig 1.

Many of the normal components of plants appear to have a low density and may reach an isopycnic position after centrifugation in a 0–40% sucrose column The exact conditions of the preceding low-speed centrifugation of a crude extract from diseased plants often has a surprisingly large effect on the results obtained in rate zonal centrifugation Dilution of the crude extract, time and speed of centrifugation, and volume in the centrifuge tube are important

Gradient columns should be centrifuged immediately after the virus solution has been floated on them If they are allowed to stand, droplet sedimentation will develop (Brakke, 1953, called "streaming effect" by Anderson 1955, 1956), and the virus will soon be distributed throughout the column

It is, of course, necessary to handle gradient columns gently both before and after centrifugation to prevent unnecessary mixing Ander-

son (1955, 1956) has stressed the importance of very gradual acceleration and deceleration during centrifugation. No special precautions are taken in the author's laboratory in operating the Spinco Model L centrifuge for density gradient centrifugation except for gradual acceleration up to a few hundred r p m On occasions, a virus zone that looks as if it had been shaken is obtained. The disturbance apparently occurs during deceleration, or as the tubes fall from a horizontal to vertical position. There is a difference between individual rotors, and with some the swinging buckets fit snugly and may stick slightly before falling

Higher virus concentrations and thicker initial zones can be used in equilibrium zonal centrifugation than in rate zonal centrifugation Because of the high viscosities of dense sucrose solutions, these gradient columns should be kept as short as possible If the gradient column plus virus solution does not fill the tubes enough to prevent their collapse, water may be floated on top of the virus solution. The virus solution will usually have a higher density than water—if it does not, a little sucrose may be added to it. The virus zone usually becomes narrower during equilibrium zonal centrifugation; a fivefold concentration is not uncommon.

D. Interpretation of Results and Removal of Samples

Virus zones may often be readily seen by scattered light if the centrifuged gradient columns are illuminated in a dark room by a narrow beam of light shining into the tube from above or below. Some microscope lamps are satisfactory, but others are too hot or cannot be focused to a narrow enough beam The visibility of the virus zone depends on the difference in light scattered from it and from the surroundings. Therefore, the column should be observed in a dark room with a beam of light that does not impinge on the sides of the tube, which are often scratched. The ease with which the virus zone can be seen depends both on the concentration of virus and on the relative concentrations of virus and impurities. There was just as much virus in tube A of Fig. 1 as in tubes C or E; the difference in visibility of the virus zones is due only to differences in concentrations of other components. If the virus is pure enough, as little as 2 μg may give a visible zone (Brakke, unpublished). An experienced observer can tell roughly the relative concentration and purity of a virus from the appearance of centrifuged gradient columns. Conducting a series of experiments with a purified virus at different concentrations (e g, tobacco mosaic virus) with and without added plant extract is the best way to learn to interpret the appearance of centrifuged columns. If a virus zone cannot be seen, it may be located

by infectivity assays of samples removed from different depths, provided an infectivity assay is available for the virus

Brakke (1953) measured the amount of light transmitted at different depths in a gradient column to obtain a scanning pattern showing the location and width of the virus zone The main disadvantage was the optical imperfections of the plastic tubes. With improvements in technique, it should be possible to obtain quantitative results on concentration and location of components after density gradient centrifugation

Several techniques have been proposed for removal of samples. The simplest is to insert either a 20-gauge, 4-in hypodermic needle with its tip bent to a 90° angle (Brakke, 1955), or a micropipette (Ottesen and Weber, 1955), to the desired depth and slowly remove a sample Most of the sample will come from the layer of liquid immediately above the tip of the needle or pipette Alternatively, the sides of the plastic tube may be punctured with a short, small hypodermic needle through which the sample is removed (Brakke, 1953)

Randolph and Ryan (1950) made a slicer for cutting through centrifuge tubes The knife blade separated the liquid into two parts, the upper of which was pipetted off Hogeboom and Kuff (1954) added a heavy sucrose solution to the bottom of the tube to float the liquid column slowly up and through a plastic disk with many small, conical holes and into a second tube That sample of liquid in the upper tube could be pipetted off with little or no mixing with the solution below the disk Anderson (1955, 1956) described a device similar in principle except that the gradient column was floated into a tube formed by a hole through each of two superimposed Lucite blocks Both Lucite blocks fitted on top of the centrifuge tube, and the holes were only slightly smaller than the inner diameter of the tube When the surface of the liquid was at the desired height, the upper block was moved slowly sideways so that the holes in the two blocks no longer coincided The portion of liquid isolated in the upper block was pipetted off Phelpstead and Roodyn (1957) used a similar device with a sliding shutter instead of sliding Lucite blocks Kolin (1955) described a special pipette for removal of samples from gradient columns This pipette was essentially a Lucite plunger with a cross-sectional area ¾ as large as that of the gradient column When the plunger was inserted into the tube, the gradient column was extended and the distances between zones increased Samples were removed through a hole bored in the plunger and emerging near its tip

Removing samples with a hypodermic needle or a micropipette is adequate if one desires representative samples from selected depths

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similar to these viruses Furthermore, wound tumor virus migrated imperceptibly, if at all, in electrophoresis at pH 6 0, whereas most of the normal components did migrate.

In many cases it is not possible to obtain highly purified preparations of plant viruses even by two successive steps of zonal centrifugation followed by zone electrophoresis, unless a selective denaturing or precipitating procedure is used. The normal colloidal particles from plants of the size range of viruses have a wide range of densities and electrophoretic mobilities These particles are surprisingly stable if they are kept cold, and unstable viruses often must be kept cold and processed quickly. The selective denaturation procedures which can be used are determined by the stability of the virus The stability of the virus often changes as it is purified, and the stability of partly purified as well as of unpurified virus must then be determined The virus must also be unaggregated for the successful application of centrifugal techniques, and particularly for density gradient centrifugation The determination of conditions necessary for dispersion and stability of a virus may be the most difficult task in its purification The importance of using an adequate assay in this part of the work cannot be overemphasized

The amount of material with the same sedimentation rate, density, and electrophoretic mobility as the virus may be very small, but the concentration of virus may be equally small Wheat streak mosaic virus, for example, could not be satisfactorily purified solely by successive application of rate and equilibrium zonal centrifugation and zone electrophoresis (Brakke, 1958b) It was necessary first to keep the extract of diseased plants at 40°C for one hour Sixty to seventy per cent of the virus was lost, but a still greater proportion of normal plant components aggregated Wheat streak mosaic virus purified from heated extracts by rate and equilibrium zonal centrifugation followed by zone electrophoresis appeared to be very pure, judged by electron microscopy or by the absence of other light-scattering components in the final purification steps These preparations, however, still contained host components serologically detectable (Moorhead, 1959)

Tomato spotted wilt virus aggregated in 0 1 *M* salt and was sedimented by low-speed centrifugation The pellet was suspended in 0 01 *M* salt, which dispersed the virus, but other components of the pellet had about the same sedimentation rates as in 0 1 *M* salt and were mostly removed by low-speed centrifugation The virus was then purified by rate zonal centrifugation followed by either equilibrium zonal centrifugation or zone electrophoresis The virus proved to be too unstable to survive application of both these last two steps consecutively.

Hills (1959) reported that tobacco mosaic virus formed arrangements

Very little contamination results if a needle is slowly passed through a virus zone If it is necessary to remove consecutive samples that will include all the liquid of the column, a hypodermic syringe or pipette is not adequate, and one of the other sampling devices should be used. There is a possibility of contamination with any procedure due to adherence of solution and adsorption of particles on the walls of the tube. The more slowly samples are removed, the less contamination there should be The pipette advocated by Kolin should increase the separation between zones, but it would also increase the danger of contamination from wall effects since there would be a larger wall area to volume ratio due to the decrease in cross-section of the column.

E Equipment

An angle rotor may be used for equilibrium zonal centrifugation and for isopycnic gradient centrifugation, but a swinging bucket rotor is preferable A swinging bucket rotor is essential for rate zonal centrifugation (Brakke, 1953). High-speed swinging bucket rotors are now offered by many manufacturers of high-speed centrifuges and preparative ultracentrifuges. Any of these should be satisfactory provided they are fast enough and cool enough for the virus with which they will be used. The necessary speed will depend on the virus. It requires approximately as much time at a given centrifugal force for rate zonal centrifugation as it would take to sediment the virus completely in ordinary centrifugation.

IV. Applications to Plant Viruses

A. Purification

Density gradient centrifugation has been used for the purification of wound tumor (Brakke *et al*, 1954), potato yellow dwarf (Brakke, 1956), tomato spotted wilt (Black *et al.*, 1952, Black, 1955), wheat streak mosaic (Brakke, 1958b), white clover mosaic (Bos *et al*, 1959), bean yellow mosaic (Bancroft and Kaesberg, 1959), and beet yellows viruses (Mundry and Schneider, 1956; Mundry, 1958). Wound tumor and potato yellow dwarf viruses were purified by rate zonal centrifugation, equilibrium zonal centrifugation, and zone electrophoresis in a density gradient column (Brakke, 1955). Bean yellow mosaic virus was purified by differential centrifugation followed by rate and equilibrium zonal centrifugation. Beet yellows virus was partly purified by a single equilibrium zonal centrifugation of clarified sap in an angle rotor. No selective denaturing or precipitating procedures were used for these four viruses. Both wound tumor and potato yellow dwarf viruses are large, and their hosts had relatively few colloidal particles of a size and density

barley stripe mosaic virus zone A visible zone was not obtained with an untreated extract of diseased barley plants (tube A) Tube B contained an untreated extract from healthy barley plants Enough normal plant components were denatured and aggregated after 1 hour at 40°C so that a visible zone was obtained (tube C) in the presence of detergent The virus aggregated after heating, but was redispersed by the detergent, 0 1% Igepon T-73 This detergent also dispersed many of the normal components, as shown by tube D Shaking the extract with *n*-butanol was even more effective in removing light-scattering impurities than was heating 1 hour at 40°C, as can be seen by a comparison of tubes C and E Observation of tubes A, E, and C shows that either an hour at 40°C. or *n*-butanol treatment would be useful in the purification of barley stripe mosaic virus Visual inspection would not be adequate to compare the merits of the two denaturing procedures Some type of quantitative measurement alone or in combination with density gradient centrifugation could be used to compare the two procedures The double virus zones in tubes C and E are believed to be due to aggregation of the virus

D. Correlation of Infectivity with Particle Size and Density

The identification of infectious plant virus particles depends on indirect means because infection has not been obtained with less than 50,000 particles of any plant virus (Steere, 1955) Zonal centrifugation has been used to show that the infectious particle has the same sedimentation rate and density as particles observed in the electron microscope for wound tumor virus (Brakke *et al*, 1954) and wheat streak mosaic virus (Brakke and Staples, 1958) With the latter virus, rods less than 650 mμ long were partly separated from longer rods and shown to be noninfectious.

Matthews (1959) reported the separation of the nucleoprotein of turnip yellow mosaic virus into two components by equilibrium zonal centrifugation in a cesium chloride gradient column After being recycled twice, the faster sedimenting component had only 3% the specific infectivity of the slower sedimenting one Since this 3% could have been due to contamination, Matthews concluded that only the lighter of the two nucleoprotein fractions was infectious Both nucleoprotein fractions gave the same ultraviolet absorption and the same nitrogen-phosphorous ratio

V Conclusion

Density gradient centrifugation is a zonal separation procedure which should be applicable to many problems requiring separations of particles

suggestive of true crystals after purification by zonal centrifugation from untreated extracts of diseased plants The crystallike arrangement appeared during drying of a droplet of virus solution on the electron microscope specimen grid after dialyses to remove sucrose.

B. Separation of Serologically Related Components from Infectious Virus

Extracts of many hosts with virus diseases contain not only the infectious virus particle but also smaller particles serologically related to the infectious particle. Black and Brakke (1954) separated a small antigen from extracts of hosts infected with wound tumor virus by density gradient centrifugation. This antigen did not sediment measurably, whereas the infectious particles sedimented almost 2 centimeters Wolcyrz and Black (1956) reported a similar small antigen in extracts of plants infected with potato yellow dwarf virus.

Preparations of some plant viruses, purified by common methods, contain noninfectious particles almost as large as the infectious particles. If the virus is spherical, these noninfectious particles may be similar to the infectious ones except that they lack nucleic acid Cosentino *et al.* (1956) separated the protein and nucleoprotein fractions of turnip yellow mosaic virus by density gradient centrifugation. Sinclair *et al.* (1957) similarly separated two components of wild cucumber mosaic virus, the lighter component of which had a sedimentation constant of 59 2*s* and the heavier component, 115 5*s*. Only the latter was infectious. Bancroft and Kaesberg (1958) obtained incomplete separation of three components with sedimentation constants of 73, 89, and 99*s* in a preparation from alfalfa mosaic virus. The slower and faster components were obtained about 90% pure by one zonal centrifugation, and the middle component was about 50% pure.

C Analysis

The potentialities of zonal centrifugation for quantitative analyses have not been developed. The scanning technique reported by Brakke (1953) is neither sensitive nor accurate enough for most purposes The approximate purity and concentration of a virus have been estimated by observing the light scattered from the centrifuged gradient columns Brakke (1956) used this technique in studies on the stability of potato yellow dwarf virus and later, (Brakke, 1958b) to determine the usefulness of different selective denaturing procedures for purification of wheat streak mosaic virus. The ease of visibility of a virus zone depends on the light scattered from the zone and from regions directly above and below, and, therefore, gives an indication of the purity of the virus.

Figure 1 shows the effects of different treatments on visibility of the

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V Conclusion

Density gradient centrifugation is a zonal separation procedure which should be applicable to many problems requiring separations of particles

according to size or density. It provides an efficient separation of comparatively small quantities of virus There is no lower limit to the concentration of virus that may be used because the superimposed density gradient prevents convection. The requirement for small quantities is a disadvantage if it is desired to purify a quantity of virus. Fortunately, large quantities of purified virus are not necessary for electron microscopy, serology, or microchemical tests.

The ability to separate low concentrations may be an advantage if the separation is a prelude to analyses or determination of sedimentation coefficients, particularly in systems where interactions and deviations from the ideal become pronounced at higher concentrations. Analysis may well prove to be the most useful application of density gradient centrifugation after instrumentation and theory have been further developed. At the present time, however, the commonest applications of density gradient centrifugation to the study of plant viruses are purification, correlation of infectivity with particle size, and identification of the infectious particle In certain situations it has proved useful for measurement of effective densities and sedimentation coefficients, for identification of viruses, and as an analytical method to assess the value of various differential denaturation procedures for purification of virus In general, density gradient centrifugation will be most useful if it is considered not as a substitute for other methods of purification and centrifugation, but rather as an additional method to complement other techniques.

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THE STRUCTURE OF SMALL VIRUSES

Dedicated to the memory of the late Rosalind E Franklin (July 25, 1920–April 16, 1958)

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I Virus Substructure and the X-Ray Diffraction Method

A. Introduction

An outstanding characteristic of most viruses that are stable enough to survive purification is that, for a given virus disease, all the individual intact particles look the same Moreover, the individual particles appear to have a well-defined structure It might be argued that these stable

viruses with regular structures are atypical, and that they have received attention out of proportion to their significance. This is, however, a rather negative viewpoint. It is more fruitful to consider viruses as a model system for studying self-replication and to study intensively a few stable, well-characterized viruses In this way we may learn how a few viruses are put together and something about their self-replication mechanism. Generalizations drawn from such detailed studies can be expected to increase our understanding, not only of the structure and function of viruses, but also of other complex biological systems. The history of the investigations on tobacco mosaic virus (TMV) during the past twenty-five years provides ample justification for this viewpoint. This review will be concerned mainly with the structural studies on small ribonucleic acid (RNA)-containing viruses, and in most detail with TMV

The pioneer X-ray investigations by Bernal and Fankuchen (1937) were among the earliest and most informative physical studies on TMV. This work was undertaken on material provided by Bawden and Pirie (Bawden *et al*, 1936) within a year of the isolation and purification of TMV by Stanley (1935). Likewise, when the first spherical virus was crystallized (Bawden and Pirie, 1938a), the preliminary X-ray investigations (Bernal *et al*, 1938) followed soon thereafter. The first X-ray photographs of TMV taken over twenty years ago demonstrated that the individual rod-shaped virus particles are built up of subunits arranged in a regular way (Bernal and Fankuchen, 1941), but this observation made little impression on the general direction of research into virus structure until fairly recently Viruses were looked at for some time rather as giant molecules Physicochemical studies were concerned largely with characterizing a virus particle in terms of its shape, size, molecular weight, and hydration, while the biochemical investigations emphasized over-all composition and chemical reactivity

*The first approach to the study of substructure in viruses by physico*chemical methods was made by Schramm (1943) who, in work published in detail in 1947 (Schramm, 1947), described the alkaline degradation of TMV. The breakdown proceeds in a stepwise fashion, yielding the small, relatively homogeneous A-protein component which can be separated from the RNA of the virus It was, however, not until fifteen years after the existence of subunits had first been demonstrated by X-rays that clear chemical evidence was provided by the end-group analyses of Harris and Knight (1952) that the protein part of TMV consists of a large number of small, chemically identical protein molecules It is now possible to identify this chemical subunit of TMV with the structural subunit "seen" by X-rays. Substructure has also been

revealed, by both biochemical and X-ray analysis, in some other small viruses which contain RNA.

It is no accident that any detailed discussion of the structure of viruses must consist largely of a review of the results of X-ray diffraction studies. Electron microscopy provides pictures which directly show the relatively large-scale morphological features of viruses that have been either dehydrated or treated in some special way. Information about over-all size, shape, and hydration can be obtained by physicochemical studies of virus solutions Biochemical studies are providing a wealth of information about the chemistry of viruses, but even knowledge of the amino acid sequence of the protein subunit and the nucleic acid linkage does not specify how the protein and nucleic acid parts of a virus are folded and assembled Investigation of the kinetics and thermodynamics of virus degradation and the reaggregation of subunits, together with the results of X-ray structure studies can clarify the problem of how the parts are assembled X-ray diffraction is at present the only method available for studying the internal three-dimensional configuration of viruses and other highly organized biological structures in the hydrated native state.

B. X-Ray Diffraction

Detailed X-ray diagrams obtained from crystalline or paracrystalline viruses tell us that these virus particles have a highly regular internal structure, and moreover give a good deal of fairly direct information about size, shape, and symmetry The information contained in these X-ray diagrams can in principle be used to determine the configuration of the polypeptide chain(s) of the virus protein subunit, the molecular arrangement of the nucleic acid part and its structural relation to the protein in the intact virus It is, however, a very long and difficult job to translate the information contained in spots on an X-ray diagram into a three-dimensional map of the distribution of matter in a virus particle. Success in this "translation" depends on performing certain chemical modifications of the material being investigated, and can be assisted by using structural information provided by other physical methods As yet, very little of this analysis has been carried out, but, nonetheless, the X-ray diffraction method has provided the bulk of our knowledge of the detailed internal structure of small viruses.

We cannot here go into the principles of X-ray diffraction techniques, and can hardly do more than mention the method of analysis The reader wishing to know more about X-ray analysis of biological structures is referred to the excellent account for the noncrystallographer by Crick and Kendrew (1957). X-ray diffraction is particularly useful for study-

ing any structure which repeats regularly in space The scale of structure which can be investigated ranges from a few angstroms, as in simple atomic and ionic crystals, to hundreds of angstroms, as in virus crystals. Special problems of technique and interpretation are presented by the larger structures since the scale is much greater than the X-ray wavelengths that it is practicable to use (~1 5 Å).

X-rays pick out the ordered part of a structure in such a way that the diffraction diagram of a substance is directly related to the structural regularity within it Disorganized material or impurities will not contribute to the ordered part of the pattern and thus will not directly affect its interpretation. The order in a structure arises from the regular arrangement of smaller parts (asymmetric units), and it is this arrangement that determines the position of the reflections ("spots") on the X-ray diagram. The intensities of the reflections are determined by the detailed internal structure of the asymmetric unit The asymmetric unit is simply related to the building block of the whole structure and may consist of any number of atoms or molecules; the important point is that the asymmetric units are structurally equivalent and all have the same environment.

C *Symmetry and Subunits in Viruses*

Crick and Watson (1956) have suggested that all small viruses are built up of protein subunits packed together in a regular manner. This hypothesis provides a simple explanation of the regular shape of small virus particles, and was based largely on the example of TMV, though there was also some crystallographic evidence for subunits in some other special cases (see Hodgkin, 1949). In another paper (Crick and Watson, 1957), reasons were presented why one might expect the small viruses to be made up of subunits The infectivity of a virus is carried by its RNA (Gierer and Schramm, 1956a,b, Fraenkel-Conrat, 1956) and the function of the protein part is presumably to provide some form of packaging for the specific RNA It appears that the RNA "codes" the virus protein, and it is reasonable to expect that the RNA may only be able to code the protein in the form of small identical molecules. Such protein subunits can then aggregate in a regular way to form the protein shell or coat of the virus.

Regular packing of a number of identical units so that the same kinds of contacts are used over and over again will result necessarily in a symmetrical structure. This means that a virus particle can be constructed out of subunits in only a limited number of ways since there are only a rather small number of kinds of symmetry possible for a particle Only rotational and translational symmetry elements can occur for a structure

built of protein and RNA, and there are fundamental geometric restrictions on the ways in which they can be combined. Symmetry will be discussed in more detail in Section III,D, but we can mention here that an n-fold rotation axis can be illustrated by a wheel with n equally spaced identical spokes, and one-dimensional translational symmetry by a ladder with equally spaced identical rungs.

The well-defined symmetry to be expected for virus particles is very favorable for X-ray structure analysis since the symmetry of the particle is indicated in a relatively direct way by the X-ray diagrams. Determination of the symmetry does not indicate what the asymmetric unit looks like. It does, however, tell us how many there are and how they are related, and thus gives some indication of the size of the structural subunit, and the over-all appearance of the virus particle.

D. *Experimental Problems and Limitations*

A physical limitation of the X-ray method is that a very large number of particles must be examined at one time in order to have enough diffracting material to obtain a photograph. For this photograph to indicate the symmetry of a single virus particle, they must be regularly arranged in space, either in a crystal for the spherical viruses, or in an orientated gel ("paracrystal") for the rod-shaped particles like TMV. The X-ray diagram of a solution or powder of randomly orientated particles gives much less information about the internal structure, but it is useful for determining the mean diameter and investigating the spherically symmetric features of "spherical" viruses.

The experimental problems involved in growing crystals of spherical viruses are discussed below (Section III,C). Rod-shaped viruses such as TMV form small true crystals in infected plants (Bawden and Sheffield, 1939) and have occasionally been observed to form unstable three-dimensionally periodic aggregates in purified preparations (Oster, 1950). The nature of the TMV structure makes it unlikely that any large stable crystals could be grown for X-ray work, but the very well orientated gels or paracrystals which can be prepared from this virus serve equally well if not better than true crystals, as indicated by the detailed X-ray results already obtained with TMV. The techniques for orientating TMV have been described by Bernal and Fankuchen (1941). The best orientation has been obtained with gels at concentrations between 15 and 25% (Holmes, 1959).

A question which often arises in connection with the X-ray study of virus structure is the effect of impurities or contaminants on the analysis. Any material which is randomly associated with the virus will not contribute to the diffraction pattern characteristic of the structurally

regular particle, but will merely affect the background of the photograph. Nonviral material which is attached to the virus in a regular way will contribute to its diffraction pattern. This kind of regular modification of the virus, rather than being a complication, actually provides one of the most powerful means of working out the detailed structure. Regular modifications, using substituents heavy enough to be "seen" by the X-rays are being sought continually, and will be discussed in more detail in relation to the interpretation of the X-ray diagrams.

A problem which the crystallographer has to face is the very weak diffracting power of viruses. The total intensity diffracted by any material in an X-ray beam is proportional to the total number of electrons irradiated by the beam. If the sample consists of regularly arranged units, the diffracted intensity will be concentrated into spots. The number of spots that can be recorded with a given X-ray wavelength is proportional to the volume of the repeating unit. Thus, a crystal of a spherical virus which has a unit cell of about 1000 times larger volume than that of a typical globular protein such as myoglobin will have about 1000 times as many spots, each having, on the average, an intensity about 1000 times less than those from the protein crystal.

Compared to the crystallography of simple organic molecules, the X-ray diffraction study of protein crystals is difficult, but it is easy to see that the problems with viruses are considerably greater. Long exposures are required, even using special high intensity X-ray tubes and other auxiliary equipment. The effect of radiation damage on the structure can be followed from the effect of increased exposure on the X-ray pattern. Fortunately for virus crystals, these effects are gradual and corrections can be made for them. For TMV the radiation damage to the structure is actually negligible. Thus it is possible to obtain diffraction patterns which refer to the structure of the virus in its native, hydrated state.

A fundamental limitation of the X-ray analysis is the resolution of the diffraction patterns, that is, the smallest spacings that can be detected. Spots can sometimes be obtained corresponding to spacings as small as 1.5 Å, but it is doubtful at present if the analysis of the X-ray patterns can be carried out with a resolution of even 3 Å. Since the distance between atoms is about 1.5 Å, it is unlikely that we will be able to determine the location of any but the very heavy ones which stand out above the relatively uniform background of other atoms. We should, however, be able to determine such significant features as the general configuration of the polypeptide chain(s) of the protein subunit and the structure and arrangement of the RNA in the virus.

E Interpretation of X-Ray Diagrams

The basic problem in X-ray structure analysis is that the diffraction pattern itself does not contain all the information necessary to determine completely the structure which gave rise to it The ultimate goal of a structure analysis is to determine the three-dimensional electron density* distribution in the asymmetric unit to a resolution compatible with the quality of the experimental data There is, however, no standard or direct method by which this can be done Each X-ray reflection or spot on the photograph corresponds to a diffracted wave which is characterized by three quantities: direction, magnitude, and phase Of these, the first two can be determined directly, but in taking the photograph, all information as to the phase angle is lost In order to reconstruct the electron density of the diffracting structure, this phase angle must somehow be determined

For simple structures, various direct methods may be used to supply the missing information, but for complicated structures like proteins and viruses, these methods are not applicable and a different approach is necessary This is the method of isomorphous replacement, first used for proteins by Perutz and his collaborators (Green *et al*, 1954) in the study of the structure of hemoglobin By binding a heavy atom, such as mercury, in a regular way to each asymmetric unit, the diffraction pattern is modified If the heavy atom does not disturb the packing and the regular structure of the protein molecules, that is, if the original structure and its heavy atom derivative are isomorphous, the changes in intensity† will be due solely to the presence of the heavy atoms These changes may be used first to determine the position of the heavy atom and then to calculate the phase angles In general at least two, and preferably more, heavy atom substitutions at different sites are necessary to determine phase angles unambiguously However, certain parts of the X-ray diagram which correspond to particular projected views of the structure may have phase angles of only 0° or 180° In such a case, a reflection is characterized by a sign, either plus or minus, and a single heavy atom substitution is sufficient to determine the sign

It should be noted that for the purpose of phase determination it is not

* For most biological structures which are constituted for the most part of atoms of low atomic number, the electron density is approximately proportional to the mass density

† To obtain significant, measurable changes, it is necessary that the additional atoms be of atomic number greater than about 70, and that there be at least one of these per 10,000–20,000 molecular weight of protein

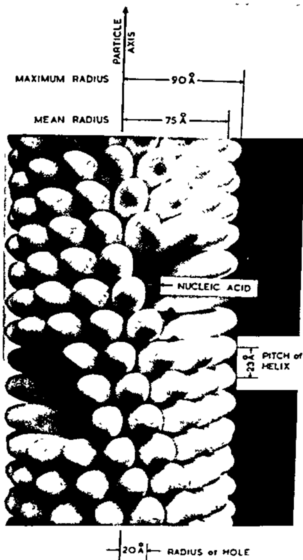


FIG. 1. A model of tobacco mosaic virus, based mainly on the X-ray diffraction studies of Rosalind Franklin (Franklin *et al.*, 1959). About one-tenth of the total length of the virus particle is shown.

The virus protein is in the form of a large number of small equivalent subunits set in helical array about the particle axis. The structure repeats after 69 Å in the axial direction, and the repeat contains 49 subunits distributed over 3 turns of the flat, major helix of pitch 23 Å. The shape of the subunits as represented is rather schematic, but is such that the helical array has a hollow core of diameter 35–40 Å

necessary to know the chemical nature of the binding of the heavy atom to the protein, all that is required is that it be specific, and that it does not significantly change the structural arrangement of the protein

Although it now appears that the method of isomorphous replacement affords the only hope for determining the detailed internal structure of a virus, a great deal of structural information can, nevertheless, be obtained by fairly direct interpretation of the X-ray diagrams Determination of the symmetry gives us considerable insight into the sub-structure of a virus, by comparing certain parts of the diagram with predictions based on simple models, some of the significant morphological features can be deduced, and comparison of the patterns given by different strains can also reveal general structural regularities

II The Structure of Tobacco Mosaic Virus

A Introduction

In some respects, TMV is unique It is unique in that it was the first virus to be discovered (Iwanowski, 1892), it was the first virus to be purified (Stanley, 1935), it was the first virus shown to be built up of regularly arranged subunits (Bernal and Fankuchen, 1941, Harris and Knight, 1952), and it was the first virus from which infectious nucleic acid was obtained (Gierer and Schramm, 1956a, b, Fraenkel-Conrat, 1956) In view of the fact that TMV is very stable and is one of the easiest viruses to obtain in quantity in purified form, it is not so surprising to find that it has been investigated more intensively than any other virus However, other reasons for the popularity of TMV are those fundamental biological, chemical, and structural properties which it possesses in which it is not unique It is now believed, for example, that most viruses consist essentially of an infectious nucleic acid core wrapped in a coat made up of a large number of protein subunits which may all be identical—a model suggested on the basis of studies first carried out with TMV The only significant drawback in choosing TMV as the simplest model virus is that it cannot be grown in tissue culture under the conditions that can be used for bacterial and many animal viruses Thus, although it is the first virus from which mutants have been produced in

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and each subunit appears as a protuberance at both the inner and outer surfaces, giving the particle a maximum diameter of 180 Å

The ribonucleic acid is deeply embedded in the protein array and is in the form of a single long-chain molecule which follows the line of the flat helix, at a radius of 40 Å Some of the protein subunits have been removed from the model to show the RNA, which is represented schematically by a smooth coil

vitro, (Gierer and Mundry, 1958), it has not yet been possible to carry out genetic fine structure studies with TMV. However, from the point of view of determining the molecular structure of a virus, this does not present a particular disadvantage

TMV is at present the only virus for which the X-ray structure analysis has been carried far enough for us to be able to build a model (Fig 1) which gives a reasonably realistic representation of the structure. Most of the information which has gone into the construction of this model has been obtained from the work of Rosalind Franklin Detailed structure analysis requires X-ray photographs of very high technical quality (Fig 2), and these can only be obtained from very well-orientated gels

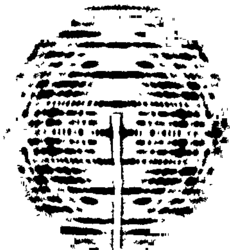


Fig 2 X-ray diffraction diagram (by Franklin and Holmes) of an orientated gel of mercury-substituted TMV, common strain, taken with a high-resolution focusing camera and crystal-monochromatized CuKα-radiation The diagram is very similar to that of TMV itself already published elsewhere (Franklin and Holmes, 1958, Franklin *et al*, 1957) but is of even higher quality

The virus particles have their long axes in the vertical direction and the pattern corresponds to the cylindrically averaged intensity diffracted by a single particle

The horizontal stratification (layer lines) corresponds to the axial repeat of 69 Å in the virus particle The intensity distribution along a layer line does not consist of discrete reflections as shown by a true crystal, but is continuous with maxima showing up as spots

The orientation of the specimen is particularly good as shown by the small degree of arcing of the spots on the diagram

B Characterization of the Virus

The TMV particle can be characterized structurally by a small set of definite numbers giving the value of certain physically and chemically measurable quantities Thus, for example, the length of the particle and the number of subunits it contains can be determined Some of these numbers can be determined more accurately than others, and at present the accuracy seems to be limited more by the precision of the experimental techniques than the variability in the properties of the virus The possibility of variation within a given virus strain does not imply that an exact numerical value is not significant For example, the mean particle length can be accurately measured even though the variation in this length may be larger than the uncertainty in the mean value We cannot hope to evaluate the significant range of variation in any quantity until we know its norm In this section we shall consider those structural properties of TMV which can be accurately measured The numerical values are summarized in Table I

1. The Helical Arrangement of Subunits

The early X-ray studies of Bernal and Fankuchen (1941) indicated that TMV has a regular substructure, but at that time the arrangement of subunits was not clear Watson (1954), using the theory of diffraction by a helix, which had been recently developed by Cochran *et al* (1952), showed that the essential features of the X-ray pattern could be accounted for if the virus particle was built up of a helical array of subunits set about the long axis of the particle In the axial repeat period of 69 A there would have to be $3n + 1$ (where n is an integer) subunits equally spaced along 3 turns of the helix Further X-ray studies by Franklin (1955a) and Franklin and Klug (1955) completely confirmed this helical structure, in particular, by showing that certain unusual features of the X-ray diagram could only be accounted for by this type of structure

a Evidence for the Helix Helical symmetry is indicated in a direct way by the X-ray diffraction pattern However, for a structure as complex as TMV, the helical parameters are by no means as obvious from its diffraction pattern as is the case for simpler helices such as DNA and α-helical polypeptides In describing the diffraction pattern of a helix, it is necessary to refer to two coordinate directions the meridian, which is the vertical axis in Figs 1 and 2 (parallel to the helix axis) and the equator, which is the horizontal axis (perpendicular to the helix axis) The diffraction pattern is symmetrical about both these directions

A smooth, continuous helix would give a diffraction pattern of intensity

TABLE I
Basic Data for TMV

Property	Quantity	Estimated uncertainty or standard deviation	*Method of measurement*
(*A*) Length of TMV particle	3000 Å	±1.7%	Electron microscopy
(*B*) Number of subunits per unit length (49.02/69.0 Å)	0.710 per Å	±0.5%	X-ray diffraction
(*C*) Number of nucleotides per subunit	3	—	X-ray diffraction + chemical analysis
(*D*) Mean molecular weight of RNA nucleotide	322	±0.2%	Chemical analysis (nucleotide composition)
(*E*) Molecular weight of TMV particle	39×10^6	±3%	Physicochemical (sedimentation-viscosity, light scattering)
(*F*) Molecular weight of protein subunit (157 ± 1 amino acids)	17,420	± 0.7%	Amino acid composition of all tryptic peptides

Derived quantities *from* basic data	Formula *from* basic data[a]	Quantity	Independently measured value[b]
Number of subunits per TMV particle	$(A) \times (B)$	2130 ± 2%	
Number of nucleotides per TMV particle	$(A) \times (B) \times (C)$	6390 ± 2%	
Molecular weight of virus RNA	$(A) \times (B) \times (C) \times (D)$	$2.06 \times 10^6 \pm 2\%$	$1.9–2.1 \times 10^6$[c]
Per cent RNA (physical)	$\frac{(A) \times (B) \times (C) \times (D)}{(E)} \times 100$		
Per cent RNA (chemical)	$\frac{(C) \times (D)}{(F) + (C) \times (D)} \times 100$	5.26 ± 1%	5.1%[d]
Molecular weight of protein subunit (physical)	$\frac{(E) - (A) \times (B) \times (C) \times (D)}{(A) \times (B)}$	17,400 ± 5%	17,420 (*F*), 17,300–17,800[c]
Molecular weight of virus particle (chemical)	$(A) \times (B) \times [(F) + (C) \times (D)]$	$39.2 \times 10^6 \pm 3\%$	39×10^6 (*E*)

Per cent phosphorus content of virus (physical)	$\frac{(A) \times (B) \times (C) \times (\text{mol wt P})}{(E)} \times 100$	0.50 ± 1%	0.45–0.56%[d]
(chemical)	$\frac{(C) \times (\text{mol wt P})}{(F) + (C) \times (D)} \times 100$		
Molecular weight per Å length of virus	$\frac{(E)}{(A)}$, $(B) \times [(F) + (C) \times (D)]$	$1.3 \times 10^4 \pm 1\%$	1.4×10^4[f]

References for basic quantities (additional references and details are given on the pages referred to in this review) (A), Williams and Steers (1951), Hall (1958), see p 242 (B), Franklin *et al* (1957), Franklin and Holmes (1958), see p 239 (C), Franklin, *et al* (1959), see p 272 (D), Knight (1951), Markham and Smith (1951), see p 250 (E), Boedtker and Simmons (1958), see p 244 (F), Wittmann and Braunitzer (1959), see p 247 and Table II

NOTE (E) or (F) is redundant since either can be calculated from the other using the basic quantities (A–D) Certain of these basic quantities could be substituted by different experimentally measured parameters, but it is felt these are the most accurately known The basis for assigning the estimated uncertainties is discussed in the text

[a] These formulas indicate the way the various derived quantities are calculated from the basic quantities The estimated uncertainties are just the sum of those of the basic quantities used in the calculation, when two different calculations give the same result, the indicated uncertainty is the smaller of the two

[b] Independently measured values are obtained by methods different from those used to obtain the calculated value The accuracy of these values are indicated in the text or original references

Method of measurement and references for independent values

[c] Sedimentation-viscosity and light scattering of extracted RNA Gierer (1957, 1958a), Boedtker (1959), see p 275 The value derived is for the acid, the sodium salt of this RNA would have a molecular weight of 2.2×10^6

[d] Phosphorus analysis of virus and RNA, Knight and Woody (1958), see p 250

[e] Sedimentation-diffusion of dissociated subunits, Wittmann (1959a), see p 245

[f] Low-angle X-ray diffraction from orientated gels, Bernal and Fankuchen (1941), see p 241

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Per cent RNA (physical)	$\frac{(A) \times (B) \times (C) \times (D)}{(E)} \times 100$	5.28 ± 1%	5.1%[d]
Per cent RNA (chemical)	$\frac{(C) \times (D)}{(F) + (C) \times (D)} \times 100$		
Molecular weight of protein subunit (physical)	$\frac{(E) - (A) \times (B) \times (C) \times (D)}{(A) \times (B)}$	17,100 ± 5%	17,420 (F), 17,300–17,800[e]
Molecular weight of virus particle (chemical)	(A) × (B) × [(F) + (C) × (D)]	39.2 × 10[6] ± 3%	39 × 10[6] (E)

teristic splitting of the layer lines The extent of this splitting varies from strain to strain of TMV, but is always quite small This splitting of the layer lines is due to the fact that the ratio of the pitch of the helix to the axial distance between neighboring subunits is not exactly a rational fraction of the form $(3n + 1)/3$ Moreover, this feature of the X-ray pattern can only be accounted for by a helical structure From the extent of the splitting, it is found that there are $(3n + 1.02) \pm 0.01$ subunits in three turns of the helix for the common strain of TMV Since n is now known to be 16, this means that from X-ray diffraction measurements it is possible to measure the number of subunits in three turns of the virus helix to an accuracy of about 2 parts in 10,000 The 69 Å value for the length of three turns is, however, only measured with an accuracy of about 5 parts in 1000

The actual number of subunits in three turns of the helix of the common strain is only about 0.04% greater than the integral value of 49 Since this difference is much smaller than the uncertainty in the measurements of other parameters for TMV, it is evident that for most purposes the integral value of 49 is sufficiently accurate

It is an interesting aspect of the X-ray diffraction studies on TMV that the difference between the actual number of subunits in three turns of the helix and an integer was determined before the value of the integer was known This is indicative both of the precision of the method and the difficulties in the analysis Usually, the number of subunits in the axial repeat of a helix is determined by measuring the translation per subunit along the axis This spacing shows up as the first nonequatorial reflection that occurs on the meridian of the X-ray diagram For TMV this spacing can now be calculated to be 69 Å/49 = 1.41 Å Since this spacing is very small, the corresponding reflection will occur in the high-angle region of the X-ray diagram where the intensity diffracted by TMV is weak and the background high Moreover, disorientation of the virus particles by even a fraction of a degree about the mean orientation position will produce enough arcing of the high-angle reflections to make it almost impossible to decide if an observed meridional reflection is truly on the meridian or slightly off it Therefore, it is not surprising that attempts to identify the meridional reflection corresponding to the axial translation per subunit were not successful

A fresh, rather unconventional approach was made to determine the number of subunits in the 69 Å repeat by Franklin and Holmes (1958) They used a mercury-substituted TMV prepared by Fraenkel-Conrat (1959) in which a methyl-mercury group is bound to the sulfur of the single cysteine residue of each protein subunit The mercury atoms serve as markers for the protein subunits and are necessarily equally

maxima and minima on a series of layer lines parallel to the equator, with a separation reciprocally related to the helix pitch. There would be no diffraction on the meridian except on the equator, and the distance of the innermost diffraction maximum from the meridian on each non-equatorial layer line increases progressively with the order of the layer line, giving a characteristic X-shaped pattern of these innermost reflections. For a very flat helix, the inner angle of this X will be very small. A helix made up of regularly spaced subunits will give rise to additional diffraction due to the subunit periodicity. For example, there will be a spot on the meridian at the high-angle spacing corresponding to the axial distance between subunits, and there will be additional layer lines if there is a nonintegral number of subunits in one turn.

Since the TMV helix is very flat (see Figs. 1 and 7), the innermost reflections on the layer lines, which are multiples of the pitch spacing (the third-order layer lines) occur very close to the meridian. In the diffraction pattern shown in Fig. 2, the inner maxima on the third-order layer lines appear to fall on the meridian, but this is because they have been overexposed in order to show up the weaker diffraction maxima. With shorter exposures they can all be shown to occur off the meridian. The pitch of the virus helix is 23 Å, from the spacings of these layer lines. Since only every third layer line has near-meridional reflections, there must be approximately an integral number of subunits distributed over three turns of the helix, to give the 69 Å repeat distance. Thus, the number of subunits in three turns is either $3n + 1$ or $3n + 2$ (it cannot be $3n$ since this would give an integral number in one turn). From measurement of the position of the innermost reflections on the non-third-order layer lines, Watson (1954) was able to show that the correct number is $3n + 1$. Direct determination of the value of n proved very difficult, and the earlier estimates were too small. The value of n is now known to be 16, so that there are 49 subunits in three turns of the helix or $16\frac{1}{3}$ per turn.

b. Number of Subunits. Franklin and Klug (1955) were able to show that the number of subunits in three turns is not an exact integer and, moreover, were able to determine by how much it differed from an integer. They observed that the intensity maxima of the TMV X-ray pattern do not, in fact, lie exactly on a set of equally spaced layer lines. Relative to the positions that the layer lines would have if there were an integral number of subunits in three turns, the layer lines of order $3n + 1$ near the center of the diagram are shifted slightly away from the equator and layer lines of order $3n + 2$ are shifted by the same displacement toward the equator. Farther from the meridian, the direction of these shifts is reversed and their magnitude doubled. This leads to a charac-

ber of subunits per unit length, as determined from the X-ray analysis of the helical structure, together with the mass per unit length determined either by X-ray or by combination of electron microscopic and physicochemical measurements. The length and molecular weight measurements of the TMV particle and the chemical determination of the subunit molecular weight will be discussed in the following sections. The relevant data for TMV are summarized in Table I. It is apparent that there is very good agreement between the results obtained by many different methods. In particular, the agreement between the physically and chemically determined molecular weight of the protein subunit provides convincing evidence that the biochemist's and crystallographer's subunits are the same.

The mass or molecular weight per unit length of TMV is obviously the weight of the particle divided by its length. The weight and length measurements must be made on monodisperse preparations to have any significance. However, the mass per unit length can also be measured by X-ray methods, which are independent of the amount of end-to-end aggregation and polydispersity of the virus preparation. From measurement of the interparticle distance in orientated gels of TMV, Bernal and Fankuchen (1941) showed that the cross-sectional area occupied by each virus particle is inversely proportional to the concentration. This indicates that the particles in the gel are distributed so as to fill the available space as uniformly as possible. Dilution of an oriented gel leads to measurable separation of the particles only in the direction at right angles to their length. The end-to-end separation of the particles is at most only a very small fraction of their length, thus the gel may effectively be thought of as consisting of long parallel chains of virus particles. The relationship between the cross-sectional area per particle and concentration found by Bernal and Fankuchen is $A = 2.36 \times 10^{-12}/C$, where A is the area in cm^2 and C the concentration in gm/cm^3. The mass per unit length of TMV is just the product of the cross-sectional area per particle and the concentration. This is 2.36×10^{-12} gm/cm or 1.4×10^4 in units of molecular weight per Å length of virus. This value applies to both the dry virus and the virus in solution (but not including water of hydration), since the measurements used to establish this value were made on both dry gels and wet gels over a range of concentrations between 50 and 13%. The value is apt to be high, however, because of the difficulties in getting an accurate measurement of the concentration of the virus from dry weights. Residual bound water, salts, and possible contaminants would all contribute to make this value high.

The molecular weight per Å length of virus can also be calculated in two other ways from different data: (a) from the molecular weight

spaced on a helix with the same axial repeat as TMV, the radius of this mercury helix is 56 Å. The distance between mercury atoms in this helix viewed end-on gives rise to a strong reflection on the equator of the Hg-TMV diagram at a spacing of about 7 Å, which is easily measurable. The relation between this reflection and the number of mercury atoms in the repeat distance can be seen as follows: the spacing between mercury atoms in this end-on view is the circumference of the 56 Å helix divided by the number in three turns. (The fact that this number is not exactly integral causes this reflection to fall slightly above and below the equator, but has no effect on the certainty with which the integral value can be determined.) With the restriction that this number is $3n + 1$, the position of this reflection leads clearly to the conclusion that there are 49 ($n = 16$) mercury atoms equally spaced along three turns of the helix. Therefore, there must be the same number of protein subunits in three turns, since there is only 1 mercury atom attached to each protein subunit.

A direct measurement of the number of subunits from the X-ray diagram of TMV itself can be obtained from a measurement of the position of near-meridional reflections. This direct determination is complicated by the fact that the protein subunits fill much of the space in the virus particle so that the periodicity along the helix does not show up equally clearly in all parts of the diagram. However, very near the outside of the virus where the ends of the subunits project and are separated by relatively large spaces filled with water (Franklin and Klug, 1956), there are large fluctuations in density which give rise to distinguishable diffraction maxima. In order to calculate the number of subunits from the position of these reflections it is necessary to know the radius of the structural feature which gives rise to them. This is the maximum radius of the particle which has been determined from the radial density map calculated from the equatorial diffraction (Caspar, 1956a). Using this radius a self-consistent interpretation of the parts of the TMV diagram due to the scattering by the outside of the particle is obtained if there are 49 structural subunits in three turns of the helix (Franklin *et al.*, 1957). Since these independent measurements refer to the structural unit of the virus, and the measurements on the mercury positions refer to the chemical subunit, it thus appears that the structural and chemical subunits are identical.

c. The Weight per Unit Length. Further confirmation of the equivalence of the TMV subunit demonstrated by chemical and by X-ray methods is provided by a comparison of the molecular weight of the structural unit as determined by physical methods with that of the chemical unit as given by end-group analysis and amino acid composition. The physical molecular weight of the subunit is obtained from the num-



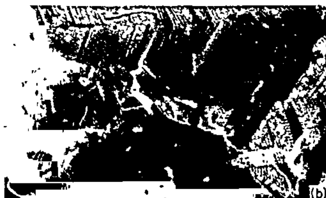


FIG 3 (a) Crystal of tobacco mosaic virus, photographed at right angles to the hexagonal face in polarized light (Wilkins *et al* 1950) The distance between dark bands is the repeating period and is approximately twice the length of the virus rods (see Fig 3b) Magnification × 3500 (Photograph supplied by M H F Wilkins)

(b) Electron micrograph of a portion of typical hexagonal crystal of TMV within a hair cell of an infected Turkish tobacco plant (Steere, 1957) Crystal cut perpendicular to hexagonal face Note the herringbone pattern due to the alternating parallel arrangement of particles within each layer, resulting in a repeat distance approximately twice the virus length Individual particles measure approximately 3000 Å in length Magnification × 50,000 (Photograph supplied by R L Steere)

in giving a length of about 3000 Å The number-average length obtained from the two most careful sets of measurements, those of Williams and Steere (1951) and of Hall (1958) are 2980 Å and 3020 Å, respectively

divided by the length of the monomeric particle, and (b) from the number of subunits per unit length times the molecular weight of the protein subunit plus its associated RNA. These both give a result of about 1.3×10^4 avograms*/Å (see Table I). The difference of about 7% between this value and that obtained from the interparticle distance in gels is within the experimental uncertainty of the measurements. It is thus clear that the weight per unit length of the virus can be obtained without assumptions regarding the unique size of the TMV particle.

2. Size of the Virus

Since the rod-shaped TMV particles are both easily broken and aggregated in the course of purification, it is difficult to obtain good monodisperse preparations of the virus. This has led, until recently, to some uncertainty about the molecular weight of the virus, and even to considerable controversy as to whether or not the virus particles have a well-defined length.

a. Length. The objections to the idea that at least most of the completed virus particles formed by an infected cell have a unique length have been based on the difficulty of preparing monodisperse TMV. It has been known for some time, however, that a substantial portion of the virus in infected tobacco cells is present in the form of crystalline hexagonal plates with well-developed faces (Bawden and Sheffield, 1939). As pointed out by Bernal and Fankuchen (1941), the virus particles could only form these true crystals if they all have the same length.

The very thorough study of these crystals *in situ* in infected hair cells of tobacco by Wilkins *et al.* (1950) (see Fig. 3a), using the polarizing microscope, has provided a clear demonstration of the unique particle length. These workers showed that the virus particles are arranged in layers parallel to the hexagonal face of the crystals. In each layer, the particle axes are parallel to each other but slightly inclined to the crystal axis; alternate layers have the particles inclined in opposite directions to give a zigzag arrangement of palisades of virus particles. This palisade arrangement has been observed directly in electron micrographs of replicas of these inclusion crystals (Steere, 1957) (Fig. 3b). From measurements with the polarizing microscope of the width of the bands seen in the TMV crystals *in situ*, Wilkins *et al.* (1950) deduced a particle length of about 3000 Å, in agreement with the value obtained from replicas of the frozen crystals in the electron microscope (Steere, 1957).

A large number of electron microscope measurements of the length of dispersed TMV particles (Oster and Stanley, 1946; Oster *et al.*, 1947b; Knight and Oster, 1947; Williams and Steere, 1951; Hall, 1958) all agree

* The unit of molecular weight is properly referred to as one avogram.



(a)

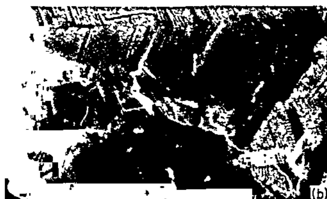


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Watanabe and Kawada, 1953) and earlier light-scattering measurements (Oster *et al*, 1947a; Oster, 1950)

The molecular weight of the subunit is the molecular weight of the *protein in the virus divided* by the number of subunits in the 3000 A long particle Since about 5 1% of the virus is RNA (see p 250), the rest being protein, the molecular weight of the 2130 subunits in the virus is $37\,0 \times 10^6 \pm 3\%$ Thus, the molecular weight of the subunit is $17{,}400 \pm 5\%$ This physically determined value is an excellent agreement with the molecular weight obtained from the amino acid composition (see Table I) Further confirmation of this value is provided by physicochemical measurements on the dissociated subunit in solution

The strong tendency of native TMV protein to aggregate in buffer solutions in which it is stable makes it difficult to characterize the subunit However, when denatured by good protein solvents such as urea (Lauffer and Stanley, 1943) or sodium dodecyl sulfate solutions (Schachman and Hersh, quoted by Fraenkel-Conrat and Singer, 1954) the subunits can be obtained in a completely dispersed state Anderer (1959) and Wittmann (1959a) have measured the sedimentation and diffusion of soluble denatured protein subunits in different solvents and have obtained molecular weights in the range 17,000–19,000 By blocking the single sulfhydryl per subunit which could produce disulfide dimers of the denatured protein, Wittmann (1959a) determined a value of 17,300 in *pyridine-water solution, in 67% acetic acid, which disperses but does not* denature TMV protein (Fraenkel-Conrat, 1957), he obtained a subunit molecular weight of 17,800 At very low protein concentration (0 01%) in neutral buffer solution, the protein is also disaggregated to the state of separate individual subunits (Ansevin and Lauffer, 1959), but accurate molecular weight measurements have not been made under these conditions

3 Chemical Composition of TMV

TMV consists of only protein and RNA (Bawden and Pirie, 1937a, Knight, 1954) and the counter-ions required for electrical neutrality No other material has been detected in purified virus preparations in significant or reproducible enough amounts to be seriously considered an intrinsic part of the virus particle

a The Protein Subunit The molecular weight of the TMV protein subunit will be unambiguously determined when its amino acid sequence is established The sequences of nine peptides (Niu and Fraenkel-Conrat, 1955b, Braunitzer, 1955 Narita, 1958a, Ramachandran and Gish, 1959, Gish, 1959), accounting for about one-third of the protein molecule, and the amino acid composition of all the peptides produced by tryptic diges-

The standard deviation of Hall's (1958) measurements is ±50 Å He remarked that this variation in the measured length may not be due to experimental errors, but may represent the actual polydispersity in the monomer length of the virus. His measurements are of particular interest since they were carried out on a purified monodisperse preparation of TMV that was extensively characterized by physicochemical methods by Boedtker and Simmons (1958) The length obtained by light scattering from solutions of this monodisperse TMV preparation is in substantial agreement with the electron microscope measurements It should be noted that the light-scattering measurement of the length is practically independent of the exact shape of the virus for a particle with as high an axial ratio as TMV

Hydrodynamic measurements (e g, flow birefringence, sedimentation and viscosity, electric birefringence) although obtained with high precision, all give consistently high values for the particle length. Boedtker and Simmons (1958) have pointed out that these length calculations require the assumption that the virus particle can be represented as an ellipsoid of revolution It is not surprising that this is a poor approximation to a helically grooved and hollow rod and that the computed dimensions of the "hydrodynamically equivalent ellipsoid" are significantly different from those of the virus

A final point in establishing the length of the virus is the possibility that the particles may shrink when dehydrated for the electron microscope Bernal and Fankuchen (1941) and Franklin (1956a) were unable to observe any shrinkage along the particle length, as shown by the constancy of the axial repeat distance measured by X-rays for wet and dry gels Moreover, the length determined by light scattering from dilute solutions is in good agreement with that found from electron microscopy. Therefore, we may reasonably take the length of 3000 Å for TMV in solution as accurate to within ±50 Å

Since there are 49 subunits in a 69 Å length of the virus, there are $49/69 \times 3000 = 2130 \pm 2\%$ in the 3000 Å long virus particle The uncertainty in this value is due largely to the uncertainty of the exact length since the X-ray measurements are accurate to about ±0 5%

b Physicochemical Molecular Weight Determination Since the particles have a well-defined length they should also have a well-defined mass The most reliable measurement of the molecular weight of TMV is that of Boedtker and Simmons (1958), based on light-scattering and sedimentation-viscosity measurements of a virus preparation very well-characterized as monodisperse Their value is $39\,0(\pm 1\,2) \times 10^6$, which is in good agreement with earlier values based on sedimentation and diffusion measurements (Lauffer, 1944; Schramm and Bergold, 1947;

Watanabe and Kawada, 1953) and earlier light-scattering measurements (Oster *et al*, 1947a, Oster, 1950)

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tion (Wittmann and Braunitzer, 1959) have already been published At the present rate of work in this field, the complete amino acid sequence of the protein may soon be established. The biochemical studies on TMV indicate that the virus protein is all in the form of subunits of molecular weight about 17,400 which appear to be chemically identical The fact that sequence analysis of the protein has so far progressed successfully suggests that the amino acid sequence of the protein subunits of a given strain of TMV is as constant and uniform as that of insulin molecules or pancreatic ribonuclease molecules of a particular species The question of how much heterogeneity is in the sequence and composition of the molecules of a particular protein has been the subject of considerable debate The weight of experimental evidence is that whatever microheterogeneity may exist in well-defined proteins, it is small enough for the chemical formula of the biologically significant molecule to be determined

In the case of TMV protein it has been pointed out (Markham, 1959) that the virus may be built up of two very similar but distinct kinds of protein subunits Such a difference could be structural (due to the way in which the polypeptide chains fold or the subunits pack) and/or chemical (due to a difference in sequence at one or more points in the polypeptide chain) The possibility that the true subunit of TMV consists of two very similar molecules, each of molecular weight about 17,400, cannot yet be ruled out, either from the biochemical or X-ray studies Until more sensitive experiments have been performed to test this possible pairing, it will be more profitable to see how well the idea of a single kind of protein subunit can account for the properties of TMV.

The first convincing evidence for chemically identical protein subunits was provided by Harris and Knight (1952, 1955), who found that carboxypeptidase liberates only threonine from a number of strains of TMV, there is one threonine molecule liberated for every 17,000–18,000 molecular weight of virus protein

The work of the Berkeley laboratory on the amino acid composition and sequence of TMV protein has been reviewed by Fraenkel-Conrat and Ramachandran (1959) Since this review appeared, Wittmann and Braunitzer (1959) have reported the amino acid composition of all the peptides produced by tryptic digestion. Compositions of peptides corresponding to all but one of those analyzed by Wittmann and Braunitzer (1959) have been published from the Berkeley laboratory (Fraenkel-Conrat and Ramachandran, 1959) These results are summarized in Table II It is apparent that there is generally good agreement between the Tübingen and Berkeley analyses, and the detailed differences need not concern us here. Wittmann and Braunitzer find 157 amino acids in

the subunit, whereas the Berkeley tryptic peptide analyses, together with the Tubingen results for the missing peptide, give 150–153 amino acids. From a complete amino acid analysis, Ramachandran (1958) has calculated the number of amino acids as 164. Ramachandran's amino acid analysis, as well as those of Aach (1958), Newmark and Fraser (1956), and Black and Knight (1953) are, however, consistent with the composition determined by Wittmann and Braunitzer. It thus seems reasonable to assume that Wittmann and Braunitzer's results are accurate to about ± 1 amino acid.* Including the *N*-terminal acetyl group (Narita, 1958a,b) and the small difference for 20 amide groups replacing carboxyls (Ramachandran, 1958), this 157 ± 1 amino acid polypeptide has a molecular weight of 17,422 ± 0.7%. The agreement with the X-ray and physicochemical results, as can be seen from Table I, is much better than could be expected from the various estimated experimental uncertainties.

b. *Charged groups.* An additional contribution to the molecular weight of the virus, which is generally neglected, are the gegen-ions necessary for electrical neutrality. From the amino acid composition shown in Table II, the net charge at neutral pH for this protein with 20 amide groups and an *N*-terminal acetyl group would be −2. However, there are apparently two carboxyl groups involved in inter-subunit bonding which do not normally titrate (Fraenkel-Conrat and Narita, 1958) since two protons per subunit are released on disaggregation of the virus (Fraenkel-Conrat, 1957, Koshland *et al.*, 1958). In addition, one of the lysine amino groups is completely unreactive even in denatured protein (Ramachandran, 1959), and may be involved in a secondary linkage so that it may not contribute to the net charge. With these assumptions the total charge at neutral pH would be +12 from 11 guanidino and 1 ε-amino groups and −13 from 12 ionized glutamic and aspartic acid residues and 1 *C*-terminal carboxyl group. Thus the net charge per protein subunit would be −1. In the intact virus there are 3 RNA phosphate groups per subunit (see next section) but they do not contribute to the surface charge on the subunit, since the electrophoretic mobilities of the intact virus and repolymerized protein are identical (Kramer and Wittmann, 1958). The net surface charge of about −1600 per virus particle determined electrophoretically by Watanabe and Ui (1956) corresponds to about −1 per subunit. The net charge, including the RNA phosphates, should be −4, but the difference between this and the electrophoretic results may be accounted for if some of the carboxyls are on the surface of the hole down the center of the virus helix or otherwise masked. Presumably, most of the charged groups are internally neu-

* This assumption has been borne out by the most recent results cited in the additional note to Table II.

TABLE II

AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF TMV*

Amino acid	Total for all peptides[a]	Complete amino acid analysis[b]	Peptide number I	II	III[c]	IV	V	VI	VII[c]	VIII	IX[c]	X[c]	XI	XII[c]
Arginine	*11*	11	*1*	*1*	*1*	*1*	*1*	*2*	*1*	*1*	—	—	*1*	*1*
Lysine	*2*	2	—	—	*1*	—	—	—	—	—	*1*	—	—	—
Aspartic acid	18(17)	19	—	*1*	—	*1*	—	*2*	3	3(2)	2(1)	—	*2*	*4*
Glutamic acid	16(15)	17	*1*	—	*3*	—	—	—	4	—	—	*1*	*1*	6(5)
Threonine	16(15–16)	17	*1*	—	*1*	*1*	—	*1*	4	*2*	—	*2*	—	4(3–4)
Serine	16(15–16)	18	—	—	*2*	*1*	—	—	—	—	*1*	*6*	*1*	5(4–5)
Proline	*8*	8	—	—	*2*	—	—	—	1	*1*	*1*	*1*	—	*2*
Glycine	*6*	6	—	—	—	*2*	—	—	—	*1*	—	*2*	—	*1*
Alanine	*14*	15	—	—	—	—	—	*2*	3	*3*	—	*1*	*1*	*4*
Valine	*14*	14	*2*	—	*3*	—	*1*	2	1	*2*	—	*1*	*1*	*1*
Isoleucine	8(7–8)	9	—	—	—	—	—	*1*	1	—	—	—	3(2)	3(3–4)
Leucine	*12*	13	—	—	—	—	—	—	1	4(3)	—	*1*	*2*	4(5)
Tyrosine	*4*	4	—	—	—	*1*	*1*	—	—	*1*	—	—	—	*1*
Phenylalanine	8(7)	8	—	—	*1*	—	—	—	—	*1*	*2*	*1*	—	3(2)
Tryptophan	*3*	2	—	—	*1*	—	—	—	—	—	—	*1*	—	*1*
Cysteine	*1*	1	—	—	—	—	—	—	—	—	—	—	—	*1*
Ammonia	—	20	—	—	—	—	—	—	—	—	—	—	—	—
Total amino acids	157 (150–153)	164	*5*	*2*	*15*	*7*	*3*	*10*	19	19(17)	7(6)	*17*	12(11)	41(38–41)

* Adapted from table of Wittmann and Braunitzer (1959), including results summarized by Fraenkel-Conrat and Ramachandran (1959)

NOTE Complete amino acid analysis of all tryptic peptides reported by Wittmann and Braunitzer (1959). Peptide numbering is according to Wittmann and Braunitzer. Amino acid analyses, and in some cases sequences, have been reported from the Berkeley Laboratory for all peptides except VII (results summarized by Fraenkel-Conrat and Ramachandran, 1959). Where the results from the Tubingen and Berkeley Laboratories agree, the values are printed in italics; when they differ, the Berkeley value is given after the Tubingen value, in parentheses.

[a] Totals for the Berkeley analyses are computed on the assumption that the peptide they have not reported will be the same as Wittmann and Braunitzer's peptide VII.

[b] Amino acid composition deduced by Ramachandran (1958) from complete amino acid analysis of TMV protein.

[c] Peptides III and VII are linked together in the intact protein (Wittmann and Braunitzer, 1959).

[d] The composition of this peptide has not yet been reported by the Berkeley Laboratory.

[e] C-terminal peptide of protein (Gish 1959).

[f] N-terminal peptide of protein (Fraenkel-Conrat and Ramachandran, 1959).

ADDITIONAL NOTE

Since this table was prepared, considerable progress has been made in elucidating the primary structure of the protein of TMV as well as that of several mutants and related strains. Anderer *et al.* (1960) have reported an almost complete sequence for the 157 amino acid polypeptide chain and their results on amino acid composition agree exactly with those of Wittmann and Braunitzer (1959). The analysis reported by Tsugita and Fraenkel-Conrat (1960) indicates 158 amino acids in the virus protein and differs from that of Wittmann and Braunitzer (1959) only by an additional isoleucine residue.

A nitrous acid mutant of TMV analyzed by Wittmann (1959b) has an amino acid composition identical to that of the wild type, there is another nitrous acid mutant analyzed by Tsugita and Fraenkel-Conrat (1960) shows some changes in composition but no change in the total number of amino acids. Three distantly related strains have been analyzed by Wittmann (1960) and although there are considerable differences in composition, in all cases the polypeptide chain appears to consist of 157 amino acids.

TABLE II

AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF TMV*

Amino acid	Total for all peptides[a]	Complete amino acid analysis[b]	Peptide number I	II	III[c]	IV	V	VI	VII[d]	VIII	IX[e]	X[c]	XI	XII[f]
Arginine	11	11	1	1	1	1	1	2	1	1	—	—	1	1
Lysine	2	2	—	—	1	—	—	—	—	—	1	—	—	—
Aspartic acid	18(17)	19	—	1	—	1	—	2	3	3(2)	2(1)	—	2	4
Glutamic acid	16(15)	17	1	—	3	—	—	—	4	—	—	1	1	6(5)
Threonine	16(15–16)	17	1	—	1	1	—	1	4	2	—	2	—	4(3–4)
Serine	16(15–16)	18	—	—	2	1	—	—	—	—	1	6	1	5(4–5)
Proline	8	8	—	—	2	—	—	—	1	1	1	1	—	2
Glycine	6	6	—	—	—	2	—	—	—	1	—	2	—	1
Alanine	14	15	—	—	—	—	—	2	3	3	—	1	1	4
Valine	14	14	2	—	3	—	1	2	1	2	—	1	1	1
Isoleucine	8(7–8)	9	—	—	—	—	—	1	1	—	—	—	3(2)	3(3–4)
Leucine	12	13	—	—	—	—	—	—	1	4(3)	—	1	2	4(5)
Tyrosine	4	4	—	—	—	1	1	—	—	1	—	—	—	1
Phenylalanine	8(7)	8	—	—	1	—	—	—	—	1	2	1	—	3(2)
Tryptophan	3	2	—	—	1	—	—	—	—	—	—	1	—	1
Cysteine	1	1	—	—	—	—	—	—	—	—	—	—	—	1
Ammonia	—	20	—	—	—	—	—	—	—	—	—	—	—	—
Total amino acids	157 (150–153)	164	5	2	15	7	3	10	19	19(17)	7(6)	17	12(11)	41(38–41)

* Adapted from table of Wittmann and Braunitzer (1959), including results summarized by Fraenkel-Conrat and Ramachandran (1959).

4 *Summary of TMV Constants*

Certain parameters for the virus particle are very exactly fixed by the X-ray analysis If we combine these with only a few of the parameters determined by chemical or physicochemical methods, we can calculate all the "constants" necessary to characterize the TMV particle Some examples of such calculations, using 6 basic constants,* are given in Table I Of the various basic constants, the largest uncertainty at present is about ±3% in the molecular weight of the virus, obtained by physicochemical methods, but this molecular weight can be calculated with equal accuracy from the other 5 constants The small uncertainty in the subunit molecular weight will be greatly reduced when the amino acid composition is definitely established Thus, the accuracy with which the typical TMV particle can be described will be limited by uncertainty in the particle length measurements

C *Morphology and Internal Structure of TMV*

1. *Diameter of the Virus Particle*

The diameter of the TMV has for some time been quoted to be 152 Å, based on the X-ray measurements (Bernal and Fankuchen, 1941) of the interparticle distance in dry-orientated TMV preparations Interparticle distances measured by the electron microscope agree well with this figure, although observers had occasionally been puzzled by the fact that isolated particles seemed to be thicker (Williams, 1957) The maximum diameter of the particle is, however, a good deal greater than 152 Å and this figure represents the packing distance between particles intermeshed to form a close-packed hexagonal array

This conclusion was reached by Franklin and Klug (1956) by comparison of the observed distribution of intensity on the third layer line of the TMV X-ray diagram with that calculated for simple, helically grooved models Significant features of the diffraction on the third layer line can be accounted for by a helically grooved model with a mean radius of 77 Å, but with a maximum radius of about 85–90 Å Moreover, the changes produced in the low-angle part of the diagram when the TMV gel is dried are consistent with the intermeshing of these helically grooved or serrated particles when they are close packed The helical grooves provide a formal description of the spaces between subunits

* Only 5 basic constants are needed Either the molecular weight of the particle, or that of the protein subunit (*E* or *F* in Table I) could be left out, since either can be calculated from the other 5 basic constants Both are included to emphasize the agreement between physical and chemical methods of characterizing TMV

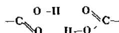
tralized so that the number of gegen ions should be small at neutral pH (perhaps about 4 per subunit) If these are sodium ions they would contribute less than 1% to the weight of the virus.

c RNA Content Values from 0 45 to 0 56% have been reported for the phosphorus content of TMV (Bawden and Pirie, 1937a; Black and Knight, 1953, Cooper and Loring, 1954, Pirie, 1956). The most extensive set of analyses of the phosphorus and nucleic acid content of TMV are those recently published by Knight and Woody (1958). From 19 different TMV preparations (including a 20-year-old one), they obtained an average phosphorus content of 0 45 gm per 100 gm of dry purified virus In the same paper, the phosphorus content of the sodium salt of the extracted RNA is reported to be 8 3% After correcting this latter value for the measured sodium content, the amount of RNA in the virus can be obtained by dividing the phosphorus content of the virus by the phosphorus content of the RNA, this is 5.1%

The number of nucleotides in the virus can be calculated from the per cent RNA, the molecular weight of TMV, and the average molecular weight of a nucleotide residue The proportions of adenine, guanine, cystosine, and uracil are 0 29 0 26 0 18 0 27 on a molar basis (Knight, 1954, Markham and Smith, 1954) and the mean molecular weight of a nucleotide residue for RNA of this composition is 322 3 The uncertainty in this value is less than ±0 2% since even a few per cent error in the relative proportions of the nucleotides has very little influence on this average The molecular weight of the RNA in the virus is 5 1% of 39×10^6 or $2 0 \times 10^6$ This is in excellent agreement with the values $1 9–2 1 \times 10^6$ obtained by physicochemical measurements on the sodium salt of isolated RNA (Gierer, 1957, 1958a, Boedtker, 1959) From the molecular weight of the RNA divided by the mean molecular weight of a nucleotide residue, there are about 6200 nucleotides in one virus particle, and since there are 2130 protein subunits, this works out to about 2 9 nucleotides per subunit As discussed below (p 272), the X-ray results show that there are an integral number of nucleotides associated with each subunit, thus this calculation indicates that 3 is the best integral value

There is a variety of ways in which the number of nucleotides per subunit can be calculated, using various combinations of chemical and physicochemical data All these various combinations of relevant experimental data agree in giving values in the range 2.7–3 2 for the number of nucleotides per subunit Although the experimentally calculated values differ by as much as 10% from the integral value of 3, there is no doubt that this integral value is the correct one for a regularly assembled complete particle of TMV

fit with the number of protons released on disaggregation, it could be postulated that the carboxyl hydrogen bonds are of the form



and that the subunits form such bonds only in successive pairs. This type of model would predict two protons released for each lead bound, but only one lead atom bound at each site for a pair of subunits. In addition, this would predict a whole new set of weak diffraction maxima for the lead-substituted virus on a set of layer lines halfway between those of the normal TMV X-ray diagram. The fact that these reflections were not observed (Caspar, unpublished) does not rule out this model, but it makes it improbable.

The limited lead binding at the 25 and 84 Å radius sites could be explained if there are other carboxyl groups at the surface of the virus which can also bind lead but with lower affinity. Gurd and Murray (1954) have obtained evidence with serum albumin that any carboxylate group capable of binding a proton can also bind lead, and in this case as many as 70 lead atoms can be bound to the carboxyls of the serum albumin molecule. In the case of TMV the two postulated carboxyls involved in inter-subunit bonding would have to have an exceptional affinity for lead; however, once the concentration exceeds some limiting value there would be a finite probability that lead may form a cross-link between two particles, leading to the observed agglomeration of the virus gel. This would imply that the apparent saturation at about half a lead atom per site is a statistical limitation due to a competitive reaction and does not have a structural basis.

8. Radial Density Distribution

The determination of the two positions at which lead binds to TMV, regardless of the chemical nature of the binding, made possible a definite determination of the signs of those equatorial diffraction maxima which correspond to the cylindrically averaged electron density of the virus (Caspar, 1956a). These signs agreed with a tentative assignment based on the consideration of the restrictions imposed on the combination of signs by the maximum diameter of the particle and by the fact that the density is everywhere positive (Caspar, 1955). Using these signs and the observed magnitudes, the Fourier transform was calculated to give the cylindrically averaged radial density distribution in the particle. The result is shown in Fig. 4a.

near the outside of the virus. That the virus surface should consist of a helical array of protuberances is reasonable because it is hard to imagine the protein subunits being of such a shape that, when helically linked, they would fill space to form a smooth cylindrical particle. This point is clearly illustrated by the model shown in Fig. 1.

The fact that the particle has a maximum radius greater than 76 Å has been definitely shown by the radial density distribution (Caspar, 1956a) determined by the method of isomorphous replacement as described below. The best estimate of the maximum radial extension of the particle comes from Franklin's unpublished work on the binding of osmium to TMV. The osmium atoms are attached to TMV at a radius of 90 Å, indicating that there is still some protein at this radius.

2. Lead-Substituted TMV

To obtain more information on the structure it is necessary to resort to isomorphous replacement. This was first successfully accomplished for TMV by Caspar (1955, 1956a) who was able to prepare a lead-substituted TMV. Lead acetate can be added to concentrated TMV solutions up to a concentration corresponding to about one lead atom per protein subunit, but addition of more lead causes the virus to precipitate or agglomerate. From the changes produced in the equatorial X-ray scattering, it was found that equal amounts of lead are bound at radial distances of 25 and 84 Å from the particle axis.

The nature of the lead binding to TMV has been investigated by Fraenkel-Conrat and Narita (1958). They found that each lead atom bound displaces a proton from the virus, and that the bound lead seems to stabilize the inter-subunit bonding so that splitting by either detergents or alkali proceeds at a slower rate. Since the disaggregation of the protein involves the release of two protons for each subunit removed (Fraenkel-Conrat, 1957, Koshland *et al.*, 1958) which are presumed to come from two carboxyl groups involved in inter-subunit bonding, Fraenkel-Conrat and Narita (1958) have suggested that it is the protons of these carboxyls which are replaced by lead. This, however, would not explain the observation by Caspar (1956a) that the two distinct lead binding sites were only half occupied. The two posulated hydrogen bonds between subunits would require that each subunit possess two carboxyl groups acting as proton donors and two sites acting as proton receptors. Thus a regular replacement of these protons would require that two lead atoms be bound at each subunit.

A possible interpretation of these results, suggested by Markham (1959), is that there are two different types of subunits. For this to

Caspar's allocation of signs was confirmed by a second successful application of the method of isomorphous replacement to TMV by Franklin (1956b) and Franklin and Holmes (1958), using the mercury-substituted TMV prepared by Fraenkel-Conrat (1959). One can, therefore, have complete confidence in the correctness of the radial density distribution shown in Fig 4. The small fluctuations which occur near the axis are of no significance since in this region the effect of experimental error is large. Furthermore, the small differences between the density distributions obtained by Caspar (1956a) and by Franklin (1956b) are due to minor differences in the methods of computation and not to any noticeable differences in the X-ray measurements.

The most significant features of the radial density distribution are as follows: (1) the virus particle has a hole of diameter 35–40 Å extending along the axis, (2) the maximum radius of the particle is close to 90 Å, (3) there are regions of high density at radii of 25, 40, 66, and 78 Å with intervening minima at radii of 31, 60, and 73 Å.

The axial hole of TMV is occupied by water when the virus is in solution, and the inside surface of the virus is as accessible to small molecules as the outside. When TMV is dried from solutions containing small solute molecules, these molecules fill in or line the hole, and under these conditions Huxley (1957) has shown that the hole is directly revealed in electron micrographs. The negative-staining technique of Brenner and Horne (1959), using neutralized phosphotungstic acid, also clearly shows the axial hole in TMV (Fig 5). A particularly interesting set of electron micrographs of repolymerized TMV protein using this technique has been recently obtained by Nixon and Woods (1960) (Fig 6). Not only do these micrographs reveal the hole down the axis of the particle, but for the first time in the history of TMV electron microscopy an axial periodicity of about 20–25 Å, corresponding to the pitch of the virus helix, is seen.

The hole in the virus is a rather natural consequence of the fact that the subunits cannot taper down to a wedge narrower than molecular dimensions that will fill the space near the axis of the helical array.

Electron density carries no chemical label, so the location of the RNA cannot be determined from the density distribution of TMV alone, but studies on nucleic acid-free particles described below demonstrate that the very high density region about a radius of 40 Å is due to the RNA. The other well-defined maxima in the density distribution at radii of

(d) A strain of TMV taken from Nigerian cowpea and grown in Burley tobacco plants (Bawden, 1958).

(e) The same as (d), grown in the Prince variety of French bean.

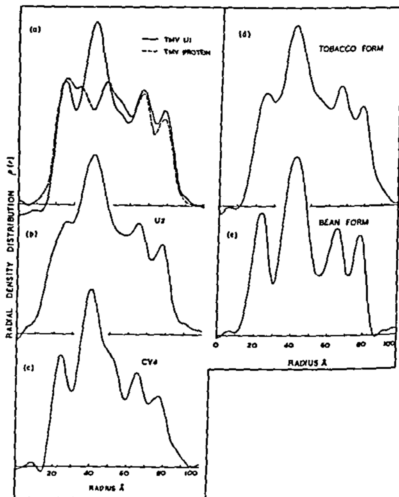


FIG 4 (a) The cylindrically averaged radial density distribution in (i) tobacco mosaic virus, common strain (full curve) (Caspar, 1956a, Franklin *et al*, 1957), (ii) repolymerized, nucleic acid-free, tobacco mosaic virus protein (dotted curve) (Franklin 1956b) The curves show the difference between the electron density of the particles and that of water, plotted as a function of radial distance from the particle axis The high peak at 40 Å in (i) is absent in (ii) and must therefore be due to the RNA in the virus The actual difference in the 40 Å region is about that expected for the 5% of RNA in the virus, taking into account the difference in the density of RNA and protein

(b)–(e) show the radial density distributions in some strains of TMV (Holmes and Franklin, 1958)

(b) Strain U2 (Siegel and Wildman, 1954)

(c) Cucumber virus 4

use of this derivative in determining the number of subunits in TMV (Franklin and Holmes, 1956, 1958) has already been described The radial distance of the mercury atom from the axis of TMV helix is 56 ± 1 Å (Franklin and Holmes, 1958), which rather precisely determines the position of the cysteine residue in the virus Since the inner and outer radii of the virus helix are about 20 and 90 Å, respectively, it is apparent that the sulfhydryl is about midway between the inner and outer surfaces of the virus This in itself can account for much of its inaccessibility, but more information about the environment of the sulfhydryl has been obtained from Franklin's (1958) further studies of the methyl mercuric derivative

To obtain more information about the protein subunit, it is necessary to investigate other parts of the X-ray diagram beside the equator However, here the diffraction amplitudes are complex so that phase angles rather than signs have to be determined, and for this purpose at least two isomorphous replacements are necessary High quality photographs (Fig 2) of the mercury derivative of TMV show significant changes in the nonequatorial reflections, but comparable photographs of the lead derivative have not yet been obtained It is thus not yet possible to determine the phase angles unambiguously * In any case it would probably be necessary to obtain still further isomorphous replacements

Some preliminary, unpublished work of Franklin on the comparison of the third layer line of the mercury derivative with that of TMV has led to the construction of a map giving a view of the electron density in TMV projected down the basic helix Since this work is based on only one isomorphous replacement, the missing elements in the deduction of phase angles were supplied by plausible arguments based on certain requirements of continuity in the X-ray amplitudes The results cannot, therefore, be regarded as established, but it does seem that certain features shown by the map are probably correct, since they are compatible with the direct interpretation of other parts of the X-ray diagram not used to construct the map (Franklin, 1958) It is found that the shape of the protein subunit is such that it protrudes at the inner as well as the outer surface The internal and external protuberances do not occur opposite one another, thus suggesting that the general lie of the protein

* Phase determination in TMV offers special problems not encountered in single crystal work for two reasons, first, the X-ray diagram represents the cylindrically averaged of the essentially three-dimensional data required to reconstruct the electron density, and second, it does not show discrete reflections as does a crystal The continuous character of the intensity distribution along layer lines does, however, offer some compensating advantages

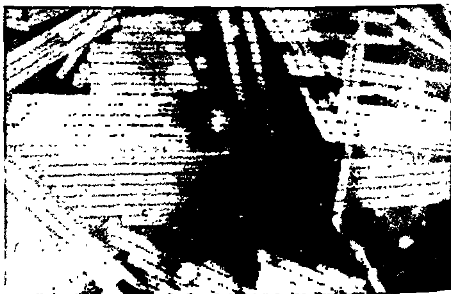


FIG. 5. Electron micrograph of tobacco mosaic virus rods "embedded" in phosphotungstic acid (Brenner and Horne, 1959). Note the axial hole down the center of the particle, and also the parallelism of the particles. Adjacent particles are never seen to interlock and the closest distance between centers is 170–180 Å. Magnification × 150,000. (Photograph supplied by R. W. Horne.)

25, 66, and 78 Å, as well as the two additional maxima which can be distinguished in the RNA-free protein particles at radii of 33 and 47 Å, must be due to the configuration of the polypeptide chain which makes up the protein subunit.

4. Structural Basis of Sulfhydryl-Masking

The sulfhydryl of the single cysteine residue in the protein subunit is not detected by the nitroprusside test unless the protein is denatured (Stanley and Lauffer, 1939). Anson and Stanley (1941) found it would react with iodine in the native virus, but with none of the other standard sulfhydryl reagents. The reaction with iodine transforms the —SH group into a stable sulfenyliodide (—SI) group (Fraenkel-Conrat, 1955). The only other reagent found to react with this unusual sulfhydryl group is methyl mercuric nitrate (Fraenkel-Conrat, 1959), and the methyl mercuric group cannot be removed even by excess thiols. Thus, even by the standards of other masked sulfhydryls, this one is unusually unreactive.

The methyl mercuric derivative prepared by Fraenkel-Conrat has made possible much of the detailed analysis of TMV structure. The

subunits is not strictly perpendicular to the particle axis, but somewhat skew to it (see Fig 1) Furthermore, there is a strong indication, substantiated by other evidence, that the shape of the protein subunits is such that their helical packing results in a set of interstitial holes in the virus particle at a radius of 55–60 Å Inner regions of the virus may be accessible to small molecules through these holes and the external grooves

The cysteine residue, as located by the position of the bound methyl mercuric group, lies close to the surface of the interstitial hole Thus small molecules or ions, such as iodine and methyl mercuric nitrate, can diffuse into this hole to react with the sulfhydryl, while large molecules such as p-chloromercuribenzoate and various thiols cannot get in Thus the spaces between subunits can be considered to be acting as "molecular sieves" which accommodate the smaller, but not the larger, molecules The interstitial holes found at a radius of 55–60 Å are presumably partly due to the packing arrangement of the subunits and partly to a crevice in the subunit, in which the sulfhydryl group is located

These structural conclusions are in agreement with the chemical behavior of the sulfhydryl group in the intact virus or repolymerized protein, on the one hand, and in the dissociated native protein, on the other (Fraenkel-Conrat, 1959) The sulfhydryl of the disaggregated protein is still masked in that it is not autoxidizable, and does not react with iodoacetate or nitroprusside until the protein is denatured However, the bound methyl mercury groups in the dissociated protein, unlike those in the intact virus or polymerized protein, can be displaced by 0 1 *M* cysteine, and the sulfhydryl can be oxidized by iodine Thus, while part of the masking is due to the structure of the protein itself, involving perhaps internal hydrogen bonding, the extreme masking in the virus is due to the almost completely buried location of the cysteine within the protein helix The implication of Fraenkel-Conrat's observations that the sulfhydryl is not involved in inter-subunit bonding has been confirmed by the X-ray studies on cucumber virus 4, discussed below (p 263)

5 *Structure of the Protein Subunit*

Very little detail regarding the structure of the subunit can be added with any certainty to what has already been described in connection with

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(2) shows a short piece of rod standing on end The number of bumps around the circle is about 16, the number of subunits per turn of helix predicted by the X-ray analysis is 16⅓ Note that the axial hole appears larger than that of short fragments of TMV (Williams, 1952) Magnification × 1,350,000

(3) Enlargement of (1) showing subunits Magnification × 1,350,000 (Photographs supplied by H L Nixon)

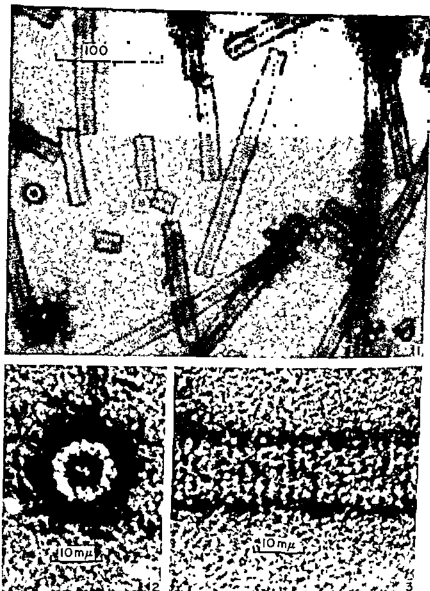


FIG 6 Electron micrographs of repolymerized tobacco mosaic virus protein (Nixon and Woods, 1960) The particles and particle fragments have been outlined with phosphotungstic acid

In (1), not only is the axial hole visible, but also a transverse striation of period about 20–25 Å This corresponds to the pitch of the TMV helix of 23 Å (although the structure may in fact be one of the variant types discussed in section II,D,4) Magnification × 270,000

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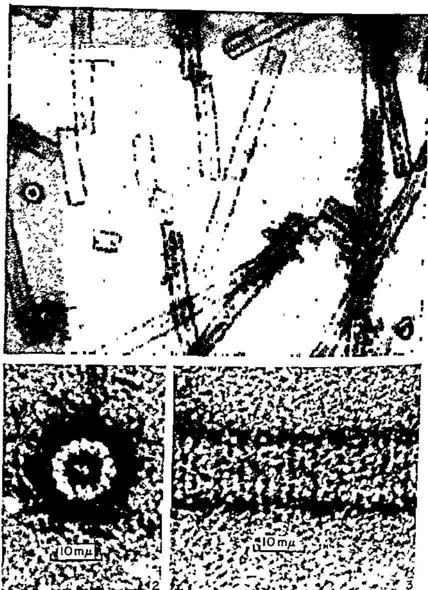


Fig 6 Electron micrographs of repolymerized tobacco mosaic virus protein (Nixon and Woods, 1960) The particles and particle fragments have been outlined

of period

(although

n H,D,β)

Magnification × 270,000

and Knight, 1952) If the lead is replacing protons involved in intersubunit hydrogen bonding there should be contact points at 25 and 84 A Contact at this large radius would imply a rather odd shape for the subunit cross-section in this region, since large enough spaces must be left for subunits of neighboring particles to interpenetrate by about 30 A when they dry together (Franklin and Klug, 1956)

Most of what has been said here about the subunit structure is highly inferential, and much of it rather vague. It is intended to indicate, however, how the limited X-ray evidence can be related to chemical and physicochemical results to obtain some idea of the detailed subunit structure.

6 Other Helical Viruses

a Viruses Morphologically Related to TMV. It was shown by Bernal and Fankuchen (1941) and later by Franklin (1956a) that different strains of TMV give X-ray diffraction patterns which bear a strong resemblance to one another, although small, measurable differences can be detected The four specimens compared by Franklin (1956a) consisted of two independent preparations of the common strain (Rothamsted strain and U1), the U2 strain (Siegel and Wildman, 1954), and cucumber virus 4 (CV4) (Bawden and Pirie, 1937b) No detectable differences were found between the two common strain preparations even though different purification techniques had been used, as might be expected from the well-defined nature of the TMV structure The patterns from the common strain, U2 and CV4 indicate that all three viruses have the same basic helical structure, but small differences in the layer line splitting (Franklin and Klug, 1955) demonstrate that each has a slightly different number of subunits in three turns of the helix. Thus, whereas the common strain of TMV has 49 02 subunits in the 69 A axial period, U2 has 49 05, and CV4 has 48 98 subunits in the same distance If all three viruses happen to have exactly the same length, these differences would mean that U2 has about one more and CV4 about two less subunits in a 3000-Å length than the common strain Obviously such small differences have little significance when it comes to counting the exact number of subunits in a virus particle, however, this clearly demonstrates that the X-ray method can detect very small differences in the way the subunits pack which would be undetectable by any other means.

Following the determination of the signs of the equatorial reflections of the common strain (Caspar, 1956a; Franklin *et al*, 1957), it became possible to deduce the signs in the different strains as well, since the diffraction patterns are so similar that it is not necessary to have a heavy atom derivative of each strain The radial density distribution

the environment of the sulfhydryl group The over-all shape of the subunit can, however, be deduced from the X-ray results. From the inside and outside dimensions of the helix (Caspar, 1956a) the length of the subunit is about 70 Å, and its average thickness from the pitch of the helix (Watson, 1954) is about 23 Å Near the outside of the helix, the grooves detected by Franklin and Klug (1956) indicate that the subunit must taper down The maximum width of the subunit at any radius can be calculated from the 22° angle of the wedge-shaped space available for it (Franklin and Holmes, 1958) Thus, at 20 Å radius the maximum width is slightly less than 8 Å The subunits appear to be hexagonally close packed in the helix out to a radius of approximately 55 Å so the width here would be about 21 Å. At larger radii the average width does not appear to increase appreciably, and must eventually taper down Thus, to a first approximation the subunit can be considered an ellipsoid of revolution with a major axis of 70 Å and a minor axis of about 20–25 Å

From the X-ray results it is clear that this is a considerable approximation In the first place there must be a significant notch in the subunit at 40 Å radius to accommodate the RNA chain which packs in a very compact way with the protein Moreover, the well-defined regions of high and low density seen in the radial density maps (Fig 4) suggest that the polypeptide chain is folded so that the backbone tends to lie in a series of cylindrical surfaces, rather than in a predominantly radial direction Watson (1954) has pointed out that the 23 Å thickness of the subunit would accommodate a double layer of α-helices The polarized infrared measurements of Fraser (1952) can be interpreted as indicating that at least part of the protein chain is folded in an α-helix, with the chain axes generally oriented perpendicular to the virus axis. Some sort of folding is, of course, required to accommodate the 157 amino acid chain in the space available, and the suggestions mentioned here are at least not inconsistent with the present limited evidence.

As more of the protein sequence is determined, and if more chemically specific heavy atom substitutions of particular amino acids become available, the location of these amino acids could be mapped in space as well as in chain position. In this way a reasonable model for the chain folding may be constructed Already we know that the cysteine residue is in the 41 amino acid N-terminal tryptic peptide (Fraenkel-Conrat and Ramachandran, 1959), which itself has considerable tendency to aggregate in filaments Thus, this region of the chain may be involved in inter-subunit bonding, which is consistent with its location in the virus particle The C-terminal end of the chain must be near the outside end of the subunit, since it is readily accessible to carboxypeptidase (Harris

unambiguous, thus there must have been unaccounted-for losses in the hydrazinolysis, or there are chemical differences in the subunits.

The most striking chemical feature of the CV4 protein is the absence of the single cysteine residue per subunit, which is characteristic of all strains of TMV (Knight, 1949, 1954) The very masked behavior of the TMV sulfhydryl (Stanley and Lauffer, 1939; Fraenkel-Conrat, 1955) could be interpreted to mean that it either is involved in a structurally important secondary linkage, or that it is just tucked safely away. Since the CV4 protein chain that contains no sulfur can fold up into a globular unit which can pack into a helix very similar to that of TMV, it is probable that the cysteine of TMV does not play a determining role in the folding or packing of its polypeptide chains

The difference in structure between the Bawden bean variant of TMV and the tobacco strain to which it is reversibly related (Bawden (1958) has been shown by Holmes and Franklin (1958) to be much greater than that between other strains of TMV and CV4 (see Fig 4). The tobacco form is very little different from common TMV, in agreement with their close biological similarity (Bawden, 1958) The host-controlled variant produced by growing this virus in bean is biologically and chemically, as well as structurally, distinct In spite of these considerable differences the bean form still has its RNA at 40 Å radius and its protein chains in the same general configuration, and similar packing arrangement as the tobacco form from which it is derived

b *Morphologically Different Viruses* The only rod-shaped virus with a structure different from the TMV helix that has been investigated by X-ray diffraction is potato virus X (PVX) (Bernal and Fankuchen, 1941) The photographs that were obtained are not as good as those from TMV, because the sinuous PVX particles (ca 5000×100 Å; Bode and Paul, 1955) are much more difficult to orientate The lack of regular side-by-side packing of the PVX particles is indicated by the absence of the low-angle interparticle diffraction effects observed with the paracrystalline TMV preparations. The photographs do suggest, however, that the structure is helical with a pitch close to that of TMV, but that the subunit packing repeats after two instead of three turns.

of CV4 was calculated in this way by both Kilkson (1957) and by Holmes and Franklin (1958), who also investigated three other strains of TMV. The results of Holmes and Franklin are given in Fig 4, and they show that the structural arrangement of protein and nucleic acid is fundamentally similar in all these strains of TMV and in CV4

The considerable structural similarity between CV4 and TMV is of particular interest, since CV4 and the closely related CV3 are unique in so many biological and chemical characteristics that Knight (1955) has concluded that these cucumber viruses are not strains of TMV. This conclusion does not rule out the possibility of a common ancestor, which is strongly suggested by the X-ray results. The ability of X-ray crystallographers to see a close relationship between biological macromolecules that may seem far apart by other criteria does not mean that structural analysis is undiscriminating Since this point is of general interest, it is worth a brief digression here. Structural similarity between biologically distinct macromolecules may indicate that the particular structure has a more permanent evolutionary significance than the particular chemical sequence or function. Thus, Perutz *et al.* (1960) have recently shown that the two chemically distinct polypeptide chains of the four-chain hemoglobin molecule have a structure very similar to one another, and, moreover, very similar to that of the one-chain myglobin molecule (Kendrew *et al*, 1959). Likewise, CV4 appears to have preserved a functionally well-adapted structure during its evolution

The earlier X-ray investigations of CV4 (Bernal and Fankuchen, 1941) showed that its packing diameter in dry gels is smaller than that of TMV by about 3% From Fig 4 it is clear that the maximum diameter is the same in each case, but that the weight of protein in the outer part of the particle is less in CV4 than in TMV. The closer packing of CV4 thus probably arises simply from a deeper interlocking of knobs and grooves.

The high density peak at about 40 Å due to the phosphate-sugar backbone in the TMV strains and CV4 shows that all have their RNA at the same radius. The correspondence of the other peaks indicates that the general configuration of the subunits is similar. Since the approximate size, number, and packing arrangement of CV4 subunits are very similar to those of TMV, their molecular weights should be comparable. Niu *et al.* (1958) have reported, however, that the number of alanine C-terminal residues per unit weight of CV4 obtained by hydrazinolysis is only about 60% of the corresponding C-terminal threonine obtained from TMV, suggesting a considerably larger subunit. This is incompatible with the X-ray results, which in this case are

(Boedtker, 1959) The denatured protein which is a by-product is generally of little use for biological or structural studies.

Splitting the virus with dilute alkali (Schramm, 1943, 1947) or with concentrated acetic acid (Fraenkel-Conrat, 1957) can be controlled to give native protein, but the RNA released under these conditions is partially degraded and noninfectious The most useful criterion that the protein is still native is its property of polymerizing at a suitable pH to form viruslike rods As shown by X-ray studies of polymerized protein, this is not an unambiguous criterion (see p 268), but it can be regarded as a necessary, if not sufficient, condition that the protein is native Viruslike protein has also been isolated from infected plants (Takahashi and Ishii, 1952, Commoner *et al* 1953, Jeener and Lemoine, 1953). It now appears that this X-protein is also native TMV protein that has not been assembled into completed virus particles (Takahashi, 1959)

2 Dissociated Protein

A-protein has been operationally defined (Schramm, 1947) as the component with sedimentation constant of about 4 6 *S* obtained by disaggregating TMV at pH 10–10 5 in low ionic strength buffers. Since the nature of this component has been clarified by recent work, A-protein can be taken to mean soluble, native, protein subunits in a limited state of aggregation, whether obtained by degrading the virus with alkali or acetic acid

a Alkaline Dissociation The action of dilute alkali to produce A-protein from TMV is a rather complicated process and it has been studied in considerable detail (Schramm, 1947, Schramm *et al.*, 1955, Harrington and Schachman, 1956) The first ultracentrifuge study (Wyckoff, 1937) seemed to indicate the occurrence of a series of well-defined components intermediate between the virus and A-protein Schramm (1947) had suggested that the dissociation proceeded by stepwise splitting into smaller fragments Harrington and Schachman (1956), however, concluded that many of the supposed intermediates were reaggregation products of the initially produced components They observed that the rate of splitting at pH 9 8 was more rapid at 0° and 40°C than at 25°C, just as with the rate of urea denaturation (Lauffer, 1943) At the lower temperature, the products seen in the ultracentrifuge are the [illegible]
twice as muc[illegible]
produced T[illegible]
is rapidly stripped from one end of the virus, but that the process stops or slows down after about two-thirds has been removed At 25°C, the

it therefore seems very likely that the virus has a loose, hollow, helical structure with a pitch slightly greater than that of TMV.

D. *Packing of the Protein Subunits*

Rod-shaped particles of TMV protein, which physically resemble the intact virus in many ways, can be prepared from the A-protein component isolated from degraded virus (Schramm, 1943, 1947) or the X-protein present in infected cells (Takahashi and Ishii, 1952). Moreover, if either of these proteins is combined with infectious TMV nucleic acid, reconstituted virus can be obtained which is physically and biologically indistinguishable from the virus produced by infected cells (Fraenkel-Conrat and Singer, 1959; Takahashi, 1959). It is the protein part of the virus that gives it its great stability and its structure is determined by the way the protein subunits pack together An individual virus particle can be considered as a one-dimensional crystal; it is periodic only in the direction of its length Like an ordinary crystal, it can be "dissolved" into its constitutive parts, and these parts can be "recrystallized" into viruslike particles

1. *Splitting the Virus*

TMV can be broken down by a great many different methods The early work with denaturing agents was principally concerned with the study of virus inactivation, but in many cases it was shown that the inactivating treatment resulted in the splitting of the protein and nucleic acid of the virus In some cases, soluble, low molecular weight protein components were observed The reagents that can disaggregate TMV include concentrated acetic acid, pyridine (Bawden and Pirie, 1937a), dilute alkali (Wyckoff, 1937), sodium dodecyl sulfate (Sreenivasaya and Pirie, 1938), urea, guanidine (Stanley and Lauffer, 1939), urethane, phenol, sodium salicylate (Bawden and Pirie, 1940) and strontium nitrate (Pirie, 1953) Heating in the presence of salt will also split the virus (Bawden and Pirie, 1937a, Cohen and Stanley, 1942). Most of these treatments result in the denaturation of the protein The denatured protein will remain soluble in the presence of the denaturing agents which are good protein solvents but will precipitate on dilution, thus separating it from the RNA

Many of the good protein-denaturing treatments fortunately have little or no effect on the integrity of the nucleic acid. High molecular weight, infectious nucleic acid has been prepared by splitting the virus with phenol (Gierer and Schramm, 1956a,b), sodium dodecyl sulfate (Fraenkel-Conrat *et al*, 1957), and heating in the presence of salt

(Holmes and Franklin, 1958). There are no other symmetry elements beside the 49/3-fold screw, and there is only one protein subunit per lattice point of the helix (Franklin *et al*, 1957). Since the protein is an asymmetric chain made up of *L*-amino acids, the part of the subunit which makes up the bottom of a turn of the helix is different from the part that makes up the top of a turn Thus, the two ends of the protein helix are necessarily different Again, since the single RNA chain which winds between turns of the protein helix (see p 272) has a sense of direction, the two ends of the RNA helix are also different.

4 Structure of Repolymerized Protein Rods

The formation of rod-shaped particles from TMV protein polymerized in the pH range 3–6 which look like TMV in the electron microscope does not necessarily mean the protein subunits are packing together exactly as in the native virus There is, in fact, X-ray evidence that variant structures can exist More striking, however, is the X-ray evidence that the same protein packing of the native virus can occur without the RNA Franklin (1955b) was able to obtain a well-oriented gel from repolymerized A-protein prepared by Schramm, which gives an X-ray diagram very similar to that of TMV itself The similarities of the two patterns indicate that the protein subunit packing is not significantly changed in the absence of RNA, and therefore the main differences between the two patterns are due to the RNA

a Native Helical Structure From the equatorial scattering of the RNA-free protein particles, Franklin (1956b) computed the radial density distribution in the protein particle This is shown in Fig 4a, where it is compared with the density distribution in the intact virus The only major difference between the two curves is that the very prominent density maximum at a radius of 40 Å in the virus is replaced by a density minimum in the RNA-free protein. This shows clearly that the RNA must be located at a radius of 40 Å The detailed information about the structure of the RNA that can be deduced by comparison of these X-ray diagrams will be considered in the next section

Certain significant features of the structure of the repolymerized A-protein are evident from its diffraction pattern (Franklin, 1955b, 1956b). The protein subunits are arranged in a helix of the same pitch and with the same number per turn as the intact virus. Moreover, the configuration of the polypeptide chains of the subunits appears to be unchanged The structure is, however, less perfect than that of TMV. This difference is indicated by a more rapid fading out of intensity maxima at large angles, and less pronounced sharp maxima in the A-protein pattern compared to that of TMV These effects can be attri-

4 S component will reaggregate to a 45 S component and the 133 S component, in turn, tends to dimerize into a 170 S component. By this sort of mechanism, most of the supposed intermediates can be accounted for.

b. Repolymerization of A-protein. In view of the observation of Ansevin and Lauffer (1959) that at very low concentration the A-protein dissociates into individual protein chains, it is probable that the stripping involves the removal of subunits one at a time. As soon as a finite concentration of subunits has been released, they will tend to associate in the 4 S equilibrium aggregate Klug and Franklin (1957) have proposed a plausible mechanism by which the 4 S A-protein component, consisting of about six subunits, can aggregate to form the stable 45 S component, rather than a continuous range of equilibrium aggregates. This 45 S component has a molecular weight of about 10^6 (Schramm *et al*, 1955) and corresponds to the disc-shaped fragments about 70 Å thickness seen in the electron microscope (Schramm and Zillig, 1955; Hart, 1955a). These discs can conveniently be built up by eight groups of six subunits or seven groups of seven subunits condensing to form three turns of the TMV helix Such a structure will have the maximum number of joined-up surfaces and thus will have a greater stabilization energy than any unclosed loop containing the same number or fewer subunits. The next stage in the aggregation would be the stacking of these three-turn helical segments to form a long segment of helix.

3. Polar Nature of the Virus

The conclusion of Harrington and Schachman (1956) that the protein is preferentially removed from one end of the virus is in agreement with the electron microscope observations of Hart (1955a) on the partial degradation of TMV with detergent. Situations have been observed where protein is removed occasionally from both ends (Hart, 1958) or the middle (Schramm *et al*, 1955) However, there does seem to be one end of the virus from which subunits can most easily be removed with or without denaturation This implied polarity of TMV is expected from the nature of its structure, and has been confirmed in one case by electric birefringence measurements

The orientation of particles of the common strain of TMV in an electric field is due to an induced dipole (Benoit, 1951; O'Konski and Haltner, 1957), but with the Holmes' rib grass strain there is an additional contribution from a permanent dipole (O'Konski and Pytkowicz, 1957). The presence of a permanent dipole definitely establishes that, for this strain, one end of a virus particle is different from the other.

From the X-ray structure studies, all strains of TMV have been found to consist of a helical arrangement of asymmetric protein subunits

(Holmes and Franklin, 1958). There are no other symmetry elements beside the 49/3-fold screw, and there is only one protein subunit per lattice point of the helix (Franklin *et al*, 1957). Since the protein is an asymmetric chain made up of *L*-amino acids, the part of the subunit which makes up the bottom of a turn of the helix is different from the part that makes up the top of a turn. Thus, the two ends of the protein helix are necessarily different. Again, since the single RNA chain which winds between turns of the protein helix (see p 272) has a sense of direction, the two ends of the RNA helix are also different.

4. *Structure of Repolymerized Protein Rods*

The formation of rod-shaped particles from TMV protein polymerized in the pH range 3–6 which look like TMV in the electron microscope does not necessarily mean the protein subunits are packing together exactly as in the native virus. There is, in fact, X-ray evidence that variant structures can exist. More striking, however, is the X-ray evidence that the same protein packing of the native virus can occur without the RNA. Franklin (1955b) was able to obtain a well-oriented gel from repolymerized A-protein prepared by Schramm, which gives an X-ray diagram very similar to that of TMV itself The similarities of the two patterns indicate that the protein subunit packing is not significantly changed in the absence of RNA, and therefore the main differences between the two patterns are due to the RNA.

a Native Helical Structure. From the equatorial scattering of the RNA-free protein particles, Franklin (1956b) computed the radial density distribution in the protein particle. This is shown in Fig 4a, where it is compared with the density distribution in the intact virus. The only major difference between the two curves is that the very prominent density maximum at a radius of 40 A in the virus is replaced by a density minimum in the RNA-free protein. This shows clearly that the RNA must be located at a radius of 40 A. The detailed information about the structure of the RNA that can be deduced by comparison of these X-ray diagrams will be considered in the next section.

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buted to a small degree of disorder in the structure of the polymerized A-protein. It is probable that this disorder results because the subunits are not so firmly fixed in place in the absence of the RNA. These observed differences between the polymerized protein and the virus are not surprising, what is surprising is that they are so small

A more marked difference is observed when the polymerized protein is dried The distance between turns of the helix shortens from 23 Å in the wet gel to about 20.5 Å in the dry gel and the structure becomes more disordered In contrast, the pitch of the helix for the intact virus is maintained at 23 Å on drying and only slight disordering results Presumably in solution the space normally occupied by RNA in the intact virus is replaced by water and anions to make up for the missing phosphate groups of RNA in the repolymerized protein. This structure is stable, but when the water is removed by drying, the particle shrinks and becomes partially disordered The RNA thus has a considerable stabilizing effect on the protein packing in the dry state, but can be replaced, at least structurally, by water and anions when the polymerized protein is in solution

b. Variant Structure The unusual X-ray patterns obtained by Franklin from the polymerized rods prepared by Commoner from the abnormal protein present in infected plants (Franklin and Commoner, 1955) provided the first indication of protein packing arrangements different from that of the virus At about the same time, Rich *et al* (1955) reported that polymerized abnormal protein prepared by Newmark gave a pattern very similar to TMV. The starting material in both cases was the X-protein (Takahashi and Ishii, 1952) but the methods of preparation differed. Repolymerized A-protein studies by Franklin (1955b), as already discussed, gave a pattern very similar to TMV, whereas repolymerized A-protein prepared and examined by Caspar (unpublished) gave a pattern corresponding to a mixture of the variant and TMV structures. Again, the starting material was the same but the methods of preparation differed These observations provide additional confirmation for the identity of the X- and A-protein already indicated by biochemical (Newmark and Fraser, 1956), physicochemical, and biological comparisons (Takahashi, 1959). It would be fortuitous enough for the two polymerized proteins to have the same normal structure if they were different, but to be able to form the same variant structure they must be quite indistinguishable proteins The variant structure most likely results from some small structural modification of the protein produced by the particular methods of purification employed

The variant rod-shaped structure in which the virus protein can also

polymerize is more poorly defined than the proper TMV helix. Not only is there more disorder in this variant structure, but there is also significantly weaker bonding between the subunits. It is very possible that the virus protein which forms these odd rods has been partially denatured so that the firm, regular contacts responsible for the TMV helix can no longer be formed. It is also possible that the packing arrangement is influenced by the conditions under which the polymerization is carried out. In spite of the limited information regarding these structures from their relatively poor diffraction diagrams, the resemblance to TMV is quite clear. Moreover, the differences observed help clarify the problem of how the subunits normally pack together in the virus.

The most striking similarity of the X-ray pattern of the variant structure to that of TMV is the approximate correspondence of the strong meridional reflections to the third-order layer lines of TMV. This means that it is built up of units of approximately the same axial thickness as the subunits which give the TMV helix its 23 A pitch. The similarity seems to end here, for in place of the off-meridional reflections with a 69 A layer line spacing, there are reflections on the meridian halfway between the strong ones (Franklin and Commoner, 1955). Franklin pointed out that this might be due to the subunits being packed in discs, stacked in pairs one above the other, to form the rod-shaped particle. The same pattern might also be accounted for by a parallel, double helix of subunits with approximately twice the pitch of TMV, although the stacked disc structure seems physically more plausible. In either case, only small positional rearrangements of the subunits relative to their normal helical arrangement in TMV are required to account for the structural change. Neither of these structures could accommodate the RNA chain which winds between the turns of the virus helix.

Unlike TMV, and to a much greater extent than repolymerized A-protein in the TMV structure, the variant structure is very sensitive to water content. The axial repeat distance increases by more than 20% in going from the dry to the wet gel. This is a change of about 5 A in the distance between turns or layers of subunits; in the wet gel the subunits are about 3.5 A further apart than in the virus. Such reversible stretching and shrinking indicate that the forces between turns or layers are quite weak. The strongest bonding between subunits would appear to be side-to-side in the helix. Evidently it is the interaction with the RNA that stabilizes the distance between subunits in the axial direction in the virus.

The transition from helix to stacked discs is quite plausible in terms of such interactions. It is also probable that a number of variant struc-

tures can occur In the dry state, the polymerized protein will be more disordered than when in solution, and may form more widely different structures An interesting example of a variant structure is seen in the dry state in the electron microscope Short disc-shaped fragments ("doughnuts") of polymerized protein seen end-on have a large hole down the middle (Hart, 1955a; Schramm and Zillig, 1955), unlike fragments of the virus (Williams, 1952). The hole down the middle of the repolymerized A-protein in its native structure (Franklin, 1956b) is, however, no different from that of the intact virus.

The repolymerized protein rods examined by Nixon and Woods (1960) may have their subunits arranged in the stacked disc structure. In their electron micrographs (Fig. 6) an axial periodicity of about 20 Å is clearly seen. The previous lack of success in resolving the individual turns of the TMV helix by the negative staining method may be accounted for by the nature of the helical structure. Since the groove on one side of the helix will tend to lie opposite the ridge on the other side, the groove, when it is filled in by the phosphotungstate, would tend to show as a more or less continuous blur in the electron microscope. In the stacked disc structure, the groove between two layers of subunits will be at the same level on both sides of the particle, and when filled in by phosphotungstate would be seen as a clear line across the particle, as in Nixon and Woods' electron micrograph.

Although the subunits may be able to polymerize reversibly either in the helical or stacked disc structures, the capacity of TMV protein to form rods that resemble the virus in the electron microscope is evidently not a clear-cut criterion that the protein is still native. This is an indication that the polypeptide chains are still in some globular configuration, but unless the packing, as indicated by the diffraction diagram, is the same as in the virus, the protein cannot necessarily be considered to be in the same state as in the intact virus.

E. Structure of the RNA of TMV

1. Configuration in the Virus

The over-all similarity between the X-ray pattern of reaggregated A-protein (Franklin, 1955b, 1956b) and intact TMV has already been discussed (p. 267) in connection with the structure of the protein part of the virus. The location of the RNA at 40 Å radius in the virus particle is indicated by the replacement of the prominent maximum in the radial density map of the virus by a density minimum in the RNA-free protein, as is shown in Fig. 4a (Franklin, 1956b). This conclusion is borne out by measurement of the relative mass difference in the 40 Å

region which corresponds in amount to the 5% of RNA in the virus. Additional confirmation that the structural difference between the virus and the RNA-free protein lies mainly at this radius comes from comparison of the nonequatorial parts of the two diffraction diagrams.

Franklin's results on the location of the RNA required a revision of the obvious interpretation of electron micrographs (Hart, 1955a; Schramm *et al.*, 1955) of partially degraded virus particles in which the exposed RNA appears to form a central core. The X-ray results refer to the intact virus in wet gel specimens which have not undergone any special treatment, and what was observed in the electron micrographs is undoubtedly a collapsed form of the RNA. A collapse would be expected to take place when the protein is removed, since it is the protein which maintains the RNA configuration in the intact virus.

The location of the RNA at a radius of 40 A does not by itself indicate how the RNA is arranged in three dimensions in the virus, nor how many chains of RNA there are (Franklin *et al*, 1957). To determine the configuration of the RNA it is necessary to compare the nonequatorial reflections of TMV and of repolymerized A-protein, and this has been done by Franklin (Franklin *et al*, 1959). From her work she was able to determine with a high degree of certainty the general configuration of the RNA in the virus.

In the radial density distribution of the RNA-free virus protein (Fig. 4a), there is a density minimum at the radius of 40 A where the RNA is located. Franklin *et al* (1957) pointed out that this minimum is no broader and no deeper than the minima at 60 and 73 A. The RNA must, therefore, be fitted in a very compact way into the structure of the virus protein. It has been shown by Hart (1955b, 1956) that, not only can a part of the protein be removed from the TMV particle while leaving the RNA apparently intact and preserving infectivity, but also that the RNA exposed by this treatment is covered up again when more TMV protein is supplied. This must mean that the RNA molecule does not run through the middle of individual protein subunits, but rather that the subunits pack together in such a way as to leave space for the RNA between them.

Some X-ray diagrams have been obtained by Franklin (see Holmes, 1959) of reconstituted TMV (Fraenkel-Conrat and Williams, 1955), made by recombining A-protein and RNA separated from the virus. Although the orientation of the specimens was not as good as that obtained in most of the TMV work described here, the X-ray patterns show that the RNA is going back into the same place, and in the same form, as in the original virus.

Since the RNA is embedded tightly between protein subunits, the

symmetry of the RNA must be the same as, or at least consistent with that of the protein subunits. The perfection of the X-ray pattern of TMV shows that both the protein and RNA have the same helical arrangement. It is, of course, the phosphate-sugar backbone of the RNA that will have the symmetry and not the sequence of bases (Crick and Watson, 1956).

Because the symmetry of the RNA conforms to that of the protein, and because of the structural identity of all the virus subunits, there must be an integral number of nucleotides associated in a regular way with each subunit A regular association of a nonintegral number of nucleotides with each subunit would produce additional X-ray reflections which are not observed; an irregular packing of the RNA, on the other hand, has already been ruled out from symmetry considerations. Because of errors in the phosphorus analysis of TMV and the possibility of contaminating nucleoprotein in the virus preparations (Pirie, 1956), it is not surprising that the analytical data do not give an exact integer ratio of nucleotides to protein subunits From the phosphorus and RNA analyses of TMV, reviewed above (p 250), it is clear that 3 nucleotides per subunit is the only integral value consistent with the experimental results

From the number of nucleotides per protein subunit, the known radial location of the RNA, and the helical parameters of the protein subunits, it is possible to predict all possible ways of forming a regular structure for the RNA, because of the restriction that the distance between successive phosphorus atoms along an RNA chain cannot be greater than 7.5 Å (The point that this kind of prediction was possible was made in 1955 by Caspar and Watson in an unpublished manuscript). With 3 nucleotides per subunit, there are only two regular ways of doing this Either there is a single RNA molecule that follows the line of the main protein helix of pitch 23 Å, or there are 16 chains of RNA running at a small angle to the particle axis The first of these models is very reasonable, but the second would require that the internucleotide separation be almost beyond the limit set by the bond length.

A comparison of the X-ray diagrams of TMV and of the RNA-free polymerized A-protein shows that the over-all pattern of the strong changes can be accounted for by the single chain structure (Franklin, 1958; Franklin *et al.*, 1959). In particular, a strong high-angle reflection on the equator observed in the virus, but not in the RNA-free protein pattern, indicates a single chain helix with 49 nucleotides in one turn (that is, 3 nucleotides per subunit) (Holmes, 1959). The 16-chain structure, on the other hand, is definitely inconsistent with many of the changes. The X-ray results are incorporated into the

model of the virus in Figs 1 and 7, where the RNA is represented as a helix of diameter 80 Å and pitch 23 Å No attempt has been made to show the detailed molecular configuration of the RNA as this is still unknown Two points are, however, clear There is only a single strand of RNA, since there is not enough material for a twin strand structure like DNA (Crick and Watson, 1954), and moreover, this strand is not fully extended since the P–P distance is only about 5 Å, compared with a possible maximum of 7 5 Å

Comparison of the birefringence of TMV with that of the repolymerized protein (Franklin, 1955b) indicates that the purine and pyrimidine bases are more nearly parallel than perpendicular to the particle axis, and this is in accord with measurements of the ultraviolet dichroism (Perutz, *et al*, 1950, Seeds and Wilkins, 1950) This arrangement is very reasonable for a single chain of RNA following a flat helix since the bases might be expected to lie approximately perpendicular to the chain direction Since the pitch of the helix is 23 Å, there is no possibility of hydrogen bonding between bases on successive turns of the helix

The single-stranded RNA configuration is well substantiated (Franklin *et al*, 1959), although the lack of perfect isomorphism between the repolymerized protein and the TMV does not allow a strict quantitative comparison of the X-ray diagrams Some small differences between the nonequatorial layer lines of the two X-ray diagrams cannot be entirely accounted for by a change at a radial distance of 40 Å, nor indeed can they be put down to any effect at a single radius This indicates that there are slight positional rearrangements of the protein subunits in the absence of RNA, so that the contribution of the RNA to the X-ray diagrams cannot be isolated completely

Our present knowledge of the configuration of RNA in the intact virus is most clearly summarized in Fig 7 In this drawing of a short segment of the virus, the last two turns of the protein helix are left off, showing the configuration the RNA would have if the protein were there The completely exposed turn of the RNA helix would obviously take up a random arrangement without the protein to hold it in place The compact packing of the RNA chain between the subunits is also quite evident There are 49 nucleotides in each turn of the helix, indicated by little discs on the chain, arranged to suggest that the plane of the bases is more or less parallel to the virus axis The separation between neighboring nucleotides is about 5 Å, and the phosphate-sugar backbone must be folded in such a way that successive groups of three nucleotides are equivalently related to each protein subunit When completely covered up by protein, the RNA would be inaccessible to large molecules such as ribonuclease, but small molecules, such as formaldehyde (Fraen-

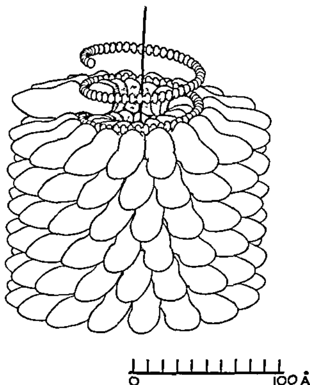


FIG 7 A drawing of the structure of tobacco mosaic virus incorporating the results of the last X-ray studies of Rosalind Franklin (posthumous paper in preparation). For dimensions and explanations, see Fig 1

For clarity, part of the ribonucleic acid chain is shown without its supporting framework of protein. it could not, of course, maintain this configuration without the protein.

There are 3 nucleotides per protein subunit, or 49 per turn of the major helix, spaced about 5 Å apart Each nucleotide is represented schematically by a flat disc parallel to the axis, since this is the predominant orientation of the purine and pyrimidine bases (Franklin, 1955b)

kel-Conrat, 1954) and nitrous acid (Schuster and Schramm, 1958), could diffuse in through the crevices between subunits to react with the RNA.

It has been reported that protein of one strain of TMV can combine with the RNA of another (Fraenkel-Conrat, 1956) and that TMV protein can even combine with synthetic polynucleotides (Hart and Smith, 1956). This would indicate that the interaction is relatively nonspecific and is primarily between the phosphate groups of RNA and basic groups in the protein However, Fraenkel-Conrat and Singer (1959) have

recently found that the combination of TMV protein with its own RNA is much more stable than with nonviral RNA. This suggests that there may be some restriction in at least part of the nucleotide sequence of the virus RNA which enables it to link up specifically with its own protein subunits

2. Structure of Isolated RNA

a Physical Properties. The X-ray diffraction method could not detect discontinuities or breaks in the covalent linkage of the RNA chain, if any were present. To learn more about the nature of the virus nucleic acid, it is necessary to look at the properties of the isolated, infectious RNA Experiments by Gierer (1957, 1958a, 1958b), which have been confirmed and extended by others (Hart, 1958, Boedtker, 1959; Cheo *et al*, 1959), have established that the infectious TMV nucleic acid is a single unit consisting of the approximately 6400 nucleotides contained within a single virus particle. Physicochemical measurement of the molecular weight of the infectious RNA is complicated by the fact that this unit is highly susceptible to degradation in the course of isolation. When TMV RNA, prepared by denaturing the protein part of the virus with phenol (Gierer and Schramm, 1956a,b), is examined in the analytical ultracentrifuge, a sharp peak representing a well-defined high molecular weight component can be observed, followed by a smaller, broad peak representing a polydisperse component of lower molecular weight (Gierer, 1957, 1958a). The amount of the polydisperse component can be increased at the expense of the high molecular weight, monodisperse component by treatments that are known to rupture the phosphodiester linkage of RNA Conversely, by taking great care to avoid conditions which would degrade the RNA during its separation from the protein, it is possible to prepare RNA which is largely in the monodisperse high molecular weight form

Even before it was recognized that the virus RNA alone is infectious, Northrop and Sinsheimer (1954) and Hopkins and Sinsheimer (1955) had prepared RNA by the heat treatment method of Cohen and Stanley (1942), taking care to avoid degradation, which gave molecular weight values about 2×10^6 as measured by light scattering Although they recognized that the RNA is inherently unstable, it is difficult by light scattering to correct for any degradation that may have taken place In [illegible]

degraded material and using the sedimentation constant of 31 *S*, Gierer

(1957, 1958a) has calculated a molecular weight of 2.1×10^6 for the monodisperse infectious RNA component This value has been confirmed by more recent physicochemical measurements (Boedtker, 1959).

The isolated undegraded RNA molecule thus accounts for all the 6400 nucleotides contained within the virus particle since this number of nucleotides add up to a molecular weight of 2.1×10^6. Within the accuracy of these molecular weight measurements, there is evidently no significant weight of protein or extraneous material associated with the isolated RNA, which is what we would expect from sensitive chemical and serological measurements showing the undetectability of any significant amount of protein in the infectious RNA preparations (Gierer and Schramm, 1956; Ramachandran and Fraenkel-Conrat, 1958).

The study of the kinetics of the degradation of the extracted RNA with ribonuclease (Gierer, 1957, 1958a) leads to the conclusion that it is a single chain, in agreement with the results of the X-ray studies of the RNA in the intact virus (Franklin, *et al*, 1959). The single strand nature of the RNA has been directly demonstrated in electron micrographs, obtained by Hart (1958), of degraded TMV examined by a method slightly different from that used in his earlier work (Hart, 1955a). A strand of fibrous material is exposed in each partially degraded virus particle, and the extrapolated length is longer than any possible on the multiple chain models of the RNA. It is interesting that this extrapolated length is about 33,000 Å, which corresponds exactly with the contour length of the RNA helix of diameter 80 Å and pitch 23 Å in the virus particle of length 3000 Å. The agreement may be purely fortuitous, for it is hard to understand why the internucleotide distance should be the same in the exposed RNA as it is in the intact virus where the RNA configuration is supported by the protein. Hart suggests that it is possible that either some denatured protein or detergent helps stabilize the exposed RNA

The well-defined size of the RNA chain accounts for the unique length of the virus particle. The helical packing of the protein subunits can repeat indefinitely and, as expected, when the protein alone is polymerized, rods with a random length distribution are formed In the plant, the conditions must be such that the protein alone will not polymerize extensively, but in the presence of the virus RNA the nucleoprotein helix can form Subunits will thus pack about the RNA until it is completely covered and then the growth of the helix will cease. *In vitro* combination of protein and RNA (Fraenkel-Conrat and Williams, 1955) can also lead to the efficient reconstitution of 3000-Å long rods, provided the RNA is undegraded (Fraenkel-Conrat and Ramachandran, 1959).

b Biological Properties The demonstration that all the RNA of the

virus can be extracted in one piece does not by itself rule out the possibility that there may be biologically significant subunits. This problem has been carefully studied by Gierer (1957, 1958b), who has shown that the RNA is infectious only if the unit of molecular weight 2.1×10^6 preserves its integrity. None of the RNA degradation products, whether produced unavoidably or intentionally, are infectious. The molecular weight of the biologically active unit can be determined from the kinetics of inactivation by RNAase digestion. It is found that splitting any one of the 6400 phosphodiester links in the RNA molecule will inactivate it. In agreement with this conclusion the target size found for X-ray inactivation corresponds to the whole RNA molecule (Buzzell *et al.*, 1956, Ginoza and Norman, 1957), and viscosity measurements on RNA isolated from irradiated virus (Lauffer *et al.*, 1956) indicate chain breaks are produced at a rate consistent with the inactivation rate. Studies by Ginoza (1958) on the kinetics of heat inactivation lead to a similar conclusion. Cheo *et al.* (1959) have confirmed that in partially degraded RNA the infectivity is only associated with the residual high molecular weight component.

Not only is a single rupture of the phosphodiester backbone lethal, but even the deamination of a single nucleotide can inactivate the RNA infectivity (Schuster and Schramm, 1958). Treatment with nitrous acid converts adenine, guanine, and cytosine into hypoxanthine, xanthine, and uracil, respectively, without any breakdown of the RNA, and the deamination of any one of 3300 nucleotides leads to inactivation. Since there are some 6400 nucleotides, 4700 of which contain amino groups in the TMV RNA, the modification of some of these is evidently nonlethal; however, some of the nonlethal deaminations can be shown to be mutagenic (Gierer and Mundry, 1958).

The weight of all this evidence confirms the concept that there are no biologically active subunits in the virus RNA. It would be difficult to prove that all the nucleotides of the 2.1×10^6 molecular weight RNA are linked by normal 3′–5′-phosphodiester bonds, but it is clear that the molecule behaves as a single long-chain polynucleotide and that any break in the chain inactivates it; moreover, the sequence of nucleotides is quite specific and alterations in the sequence are generally lethal, although some may be mutagenic or without observable effect.

The X-ray diffraction results have established that the single chain RNA molecule has a very regular helical structure in the intact virus which is determined by the packing of the protein subunits. This helical structure will certainly collapse as soon as the protein is removed. The question remains though whether the infectivity of the RNA requires the persistence of some well-defined three-dimensional configuration. Fibers

of extracted TMV RNA give an X-ray diagram indistinguishable from other RNA's (Rich and Watson, 1954) which suggests an imperfect helical structure involving irregular hydrogen bonding. Studies of the ultraviolet absorption and optical rotation of RNA also indicate that highly polymerized molecules in dilute salt solutions exist in some coiled or folded configuration stabilized by hydrogen bonds (Gierer, 1957, 1958a, Boedtker, 1959, Haschemeyer *et al.*, 1959). However, this is not a superstructure that may be subjected to irreversible denaturation; rather, it appears to be an equilibrium state determined by conditions of the medium, which can change reversibly with changes in solute conditions. The molecules may be reversibly expanded by heating for short periods or reducing the ionic strength of the solution, or the hydrogen bonds may be broken by treatment with urea. None of these treatments leads to any significant reduction of the infectivity (Gierer, 1957, 1958b; Bawden and Pirie, 1957; Ginoza, 1959; Haschemeyer, *et al.*, 1959). *Thus the infectivity of the RNA does not depend on any superstructure of the single polynucleotide strand. Therefore, the biological activity must be determined mainly or exclusively by the specific linear sequence of the 6400 nucleotides.*

III. The Structure of Spherical Viruses

"Spherical" is not the most apt description of the small viruses that are not long rods, since high-resolution electron microscopy has shown that many are symmetric polyhedra (Williams, 1954; Kaesberg, 1956). One slightly asymmetrically shaped virus has been observed (Bancroft and Kaesberg, 1958), but this may be simply a case of a very short rod. The existence of substructure in the spherical viruses, as shown by the X-ray patterns obtained from virus crystals, suggests that the virus surface will consist of a symmetrical arrangement of protuberances, which could give the virus a polyhedral appearance. The term "polyhedral" has, however, already been pre-empted to describe virus inclusions in insects which are structures of an entirely different scale. *In the absence of a more acceptable term for the small non-rod-shaped virus particles we shall continue to refer to them as spherical, with the reservation that many may not look much like smooth spheres*

X-ray studies have been made on a few of the small, stable, spherical viruses which contain as their major components only protein and ribonucleic acid. The relevant data on composition, dimensions and particle weight are summarized in Table III, which also provides a comparison of values of the diameters obtained from the X-ray work with those found by electron microscopy In all cases the diameters in the

wet (and therefore more native) state are consistently higher than in the dry state This difference is presumably due to shrinkage on drying

There are a number of other, mostly larger, viruses, which have not been studied by X-ray methods, that are of considerable interest in any discussion of virus symmetry. The structural information in these cases has largely come from electron microscopy, and we shall try to correlate these results with the structural principles which have emerged from the X-ray work.

A. Spherically Averaged Structure

The diffraction patterns from virus single crystals indicate in a relatively direct way their highly regular internal structure, but the detailed analysis of this structure is a complicated problem The X-ray scattering from dilute solutions, while providing much less information than the single crystal diffraction pattern, is much easier to interpret The spherically averaged structure can be analyzed in this way since the X-ray scattering from a dilute solution of identical particles is proportional to the intensity scattered by a single particle averaged over all rotational orientations This method has been used by Beeman and his colleagues (Leonard *et al*, 1953, Schmidt *et al*, 1954) to measure the mean diameter of a number of spherical viruses in solution

The analysis of the low-angle X-ray scattering is very similar to that for light scattering by large particles If the particles are symmetrically shaped, it is a relatively easy matter to determine their mean spherically averaged diameter by comparing the observed intensity pattern with that calculated from simple models It is also possible to calculate the spherically averaged radial density from measurements of the low-angle scattering (Fig 8) (Anderegg and Geil, 1959) This procedure is analogous to the calculation of the cylindrically averaged radial density of TMV using the equatorial scattering from oriented gels, with the significant difference that much less detail in the structure can be resolved in the spherical viruses than in TMV by this method This limitation in resolution is fundamental [illegible]

[illegible] contribution due to the higher internal symmetry In the case of TMV, however, the pure, cylindrically symmetric contribution extends to relatively high angles before the other contribution is appreciable, whereas in the case of a spherical virus, the overlap begins at much lower angles This means that structural features in the radial density

TABLE III
SPHERICAL VIRUSES

Virus	Particle weight (Avograms)	Percentage of RNA	Weight of RNA (Avograms)	Diameters by electron microscopy (in Å): Isolated particles (frozen dried)	Diameters by electron microscopy (in Å): Interparticle distance in arrays or crystals	X-ray measurements (in Å): Diameter* by low-angle scattering in solution	X-ray measurements (in Å): Interparticle distance in wet crystal	X-ray measurements (in Å): Interparticle distance in dry crystal
Tomato bushy stunt	8.9×10^{6}	17	1.5×10^{6}	300 ± 10	300	309	331 ± 9	267 ± 13
Turnip yellow mosaic (TYMV)	5×10^{6}	37	1.9×10^{6}	280 ± 10	250 260	280	306 ± 5	228
TYMV-protein particle (top component)	3×10^{6}	0	0	Much flattened[c]	—	280	314 ± 11	238
Southern bean mosaic	6.6×10^{6}	21	1.4×10^{6}	280 ± 10	245	286	295	215,250 and 207 (?)
Tobacco ringspot	ca. 5×10^{6}	34	—	260 (max 280, min 240)	250	—	—	230
Tobacco necrosis virus derivative	1.8×10^{6}	—	—	—	140	—	176,179	154,157
Poliovirus	ca. 6.7×10^{6}	25–30	ca. 1.7×10^{6}	280	272 ± 2 273 ± 1	ca. 280	304	—
Coxsackie	Probably very similar to poliovirus			280	280	—	302,310,306	—

* This is a *spherically averaged* diameter (see p. 279).
[a] Air-dried array.
[b] Air-dried crystal.
[c] Frozen replica of crystal.
[d] Dried over P_2O_5.
[e] Air-dried.

REFERENCES

[a] Cheng and Schachman, unpublished, see Hersh and Schachman (1958), Williams and Backus (1949)
[b] de Fremery and Knight (1955)
[c] Williams (1953, 1954)
[d] Kaesberg (1959)
[e] Leonard *et al* (1953)
[f] Carlisle and Dornberger (1948)
[g] Markham (1951)
[h] Cosentino *et al* (1956)
[i] Steere (1957)
[j] Schmidt *et al* (1954)
[k] Bernal and Carlisle (1948)
[l] Lauffer *et al* (1952)
[m] Miller and Price (1946)
[n] Labaw and Wyckoff (1957)
[o] Magdoff (1960), and in press
[p] Steere (1956)
[q] Bawden and Pirie (1945)
[r] Wyckoff (1950)
[s] Crowfoot and Schmidt (1945)
[t] Cowan and Hodgkin (1951)
[u] Schaffer and Schwerdt (1959)
[v] Steere and Schaffer (1958)
[w] Finch (1959)
[x] Finch and Klug (1959)
[y] Mattern and du Buy (1956)
[z] Caspar and Langridge (unpublished)

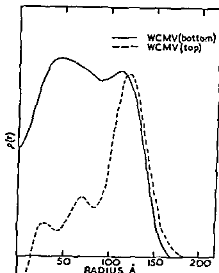


FIG 8 The spherically averaged radial density distributions of the top and bottom components of wild cucumber mosaic virus (Anderegg and Geil, 1959). The former contains no RNA, thus it can be concluded from the difference between the two curves that the RNA in the virus is centrally located and that most of the protein is in an outer shell

The virus itself (solid line) appears to have a central hole, but this and other details seen in the density distributions must be regarded with caution, because of the limited resolution, which is a good deal lower (50 Å) than that of the curves in Fig 4 (10 Å) The over-all features are, however, significant (Diagram supplied by J W Anderegg)

map of TMV that are at least 10 Å apart can be resolved whereas the resolution for spherical viruses is only about 50 Å

The low-angle scattering from a number of small plant viruses (Leonard *et al.*, 1953; Schmidt *et al*, 1954) is very similar to that of a uniform density sphere, although there is some indication of a central hole, particularly for brome grass mosaic virus (Anderegg, private communication), analogous to the axial hole of TMV. Accurate calculations of the mean particle diameters have been made which are consistent with values obtained by electron microscopy and hydrodynamic methods, if account is taken of the effect of internal substructure and hydration on the different types of measurements

For those viruses which have associated with them closely related RNA-free protein particles, for example, turnip yellow mosaic virus (TYMV) (Markham and Smith, 1949) and wild cucumber mosaic virus (Sinclair *et al.*, 1957; Kaesberg, 1959), a comparison of the low-angle X-ray scattering from the virus and protein particles provides significant

information about the distribution of protein and RNA in the virus Markham (1951) has shown by physicochemical methods that the serologically related virus and protein particles of TYMV have the same diameter and surface properties, and he suggested that in both of them the protein is in the form of a spherical shell Low-angle scattering studies (Schmidt *et al*, 1954) have confirmed that the protein particle, of mean external diameter about 280 Å, is essentially a spherical shell of mean thickness 35 Å. Very similar results have been obtained by Anderegg and Geil (1959) for wild cucumber mosaic virus by comparison of the density distributions for the top and bottom components of this spherical virus (Fig 8). Because of the limited resolution it is not possible to decide in this way if these viruses are just built up of a shell or core of RNA inside a shell of protein subunits or if the RNA is regularly arranged between protein subunits as in TMV. It is possible to conclude, however, that most of the RNA is centrally located and most of the protein goes to make up the outer shell

B X-Ray Diffraction by Virus Crystals

In order to study substructure within the protein and RNA parts of the virus it is necessary to work with single crystals The virus particles arranged in a three-dimensional crystal lattice have either all the same orientation or, at most, a small number of different, fixed orientations The diffraction pattern characteristic of a single particle is then no longer smeared out as an average over all orientations in solution, and may be effectively recorded as a three-dimensional array of crystal reflections (the reciprocal lattice)

Following the first crystallization of a spherical virus—tomato bushy stunt (BSV) by Bawden and Pirie (1938a,b—the earliest X-ray diffraction studies on virus crystals were made by Bernal and his colleagues (Bernal *et al*, 1938; Bernal and Fankuchen, 1941) They used the method of "powder" photography in which an X-ray photograph is taken of a sludge of very small, unoriented crystals in a capillary tube Despite the limited information obtained in this way, the dimensions of the unit cell of the crystal may be found and the interparticle distances and packing arrangements deduced. The first photographs of a single crystal were obtained by Crowfoot and Schmidt (1945) from a derivative of tobacco necrosis virus, and shortly afterward Carlisle and Dornberger (1948) obtained "still" photographs (see below) from single crystals of BSV However, it was not until the systematic studies of Caspar (1956b) on crystals of BSV, and of Klug *et al* (1957a,b) on crystals of TYMV that evidence regarding particle symmetry was obtained

In the last few years, X-ray photographs, of varying degrees of perfec-

tion, have also been obtained from single crystals of tobacco ringspot virus and Coxsackie virus (Caspar and Langridge, unpublished), poliovirus (Finch and Klug, 1959) and southern bean mosaic virus (SBMV) (Magdoff, 1960) These all suggest that the virus particles are built up of regularly arranged units, but only BSV, TYMV, and poliovirus have yet been studied in any detail, and it has definitely been established that these viruses possess cubic symmetry It appears likely that this is also true of SBMV Studies are most advanced in the case of TYMV, mainly because it has also proved possible to investigate the associated nucleic acid-free protein particle (Markham and Smith, 1949; Markham, 1959), but even here our knowledge is much less complete than for TMV, mainly because up to now no isomorphous replacement has been achieved. Also, there has been little chemical work on any spherical virus to compare with the huge volume of effort that has been concentrated on TMV. We shall therefore not deal with the spherical viruses in the same detail as we did TMV.

1. Experimental Techniques

A single crystal gives rise to a three-dimensional lattice of X-ray spots which is usually recorded as a series of two-dimensional sections on photographic plates To record one such section it is necessary to move the crystal in some systematic way in relation to the X-ray beam It has become the practice to use a technique in which the crystal is precessed, since this has the advantage over other techniques in that it not only gives an undistorted view of sections of reciprocal lattice but it is also possible to explore the diffraction pattern in a systematic way, from the center outward, i e., from the largest spacings to the smaller. This has the logical advantage of following the trend of the method of structural analysis which proceeds from a low-resolution picture of the virus to increasingly higher resolution

Another practice which has proved very useful is that of taking "still" photographs (Crowfoot and Schmidt, 1945) in which the crystal is kept stationary with respect to the monochromatic X-ray beam. In a crystal with a small unit cell, only very few crystal reflections would be observed, but when the unit cell is large, as in a virus crystal, many hundreds of reflections are observed, and these are arranged in sets of ellipses and circles characteristic of the lattice type (Carlisle and Dornberger, 1948). This technique has proved very valuable in the preliminary X-ray examination of virus crystals, since it gives a good deal of information in a relatively short exposure time, and is useful for setting a crystal of bad external morphology in a desired orientation Indeed the determinations of the unit cell, lattice type, and mode of particle packing in poliovirus

crystals were made solely by this kind of photograph (Finch and Klug, 1959) (see Fig 9)

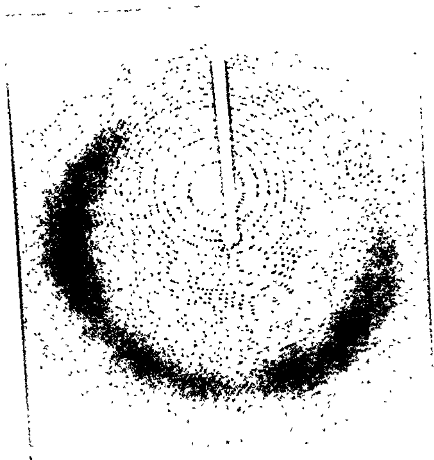


Fig 9 X-ray diffraction photograph ("still") of a crystal of poliovirus (Finch and Klug, 1959) The perfection of the crystal is shown by the presence of reflections down to spacings of 2 Å

It will by now be obvious that the X-ray techniques for studying virus crystals differ a good deal in detail from those used for TMV The orientated gels of TMV are paracrystals in which the particles are all aligned parallel, but are not otherwise fixed relative to one another

This type of arrangement is also typical of the long-chain molecules of a fiber The diffraction pattern of a paracrystalline fiber or gel represents the rotationally averaged intensity scattered by one particle rather than part of a three-dimensional pattern Instead of photographing one part at a time almost the whole of the essentially three-dimensional pattern is obtained in a single fiber diagram Thus while there is less labor involved in recording this pattern than that of a single crystal, it is not at all easy to unscramble the fiber diagram so as to obtain an idea of the three-dimensional pattern which produced it However, the X-ray study of crystalline viruses presents a number of unique problems not encountered with TMV.

2 Special Problems

The action of the X-rays is sufficient to disorder the crystals after prolonged exposure. Since the forces which hold the virus particles together in the crystal are weak compared to the forces that hold the parts of an individual particle together, the crystal structure is more sensitive to radiation damage than is the virus structure This disordering of the crystal has the effect of reducing the intensity of high-angle diffraction and hence the resolution obtainable, that is, the structure of the virus can still be "seen" but with reduced detail Generally, therefore, a fresh crystal must be used for each new exposure Under optimum conditions, X-ray reflections have been observed corresponding to spacings as small as 2 Å (Finch and Klug, 1959) (see Fig 9); this is comparable to the resolution that can be obtained from crystals of small globular proteins The net effect of the radiation damage is to limit the number of X-ray reflections that can be measured from any one crystal, but with sufficient crystals enough data could be collected to determine the significant structural details of the native virus particle

Finch (1959) has started this systematic recording for TYMV crystals but has as yet only measured 100 or so crystallographically independent reflections out to a spacing of 20 Å It should be pointed out that the task is enormous The simplest virus crystal with a unit cell side of about 350 Å would give approximately 1800 independent reflections to a spacing of 10 Å, and 14,000 out to 5 Å For more complex crystal forms the corresponding numbers would be considerably larger. Photographic methods may be inadequate for accurately measuring these large number of reflections, but it may be possible to do this using automatic counter-diffractometer techniques With any recording method, even using the highest intensity X-ray tube available, exposure times of a day or more are required to record only a very small fraction of the possible reflections given by a virus crystal. This is because the average inten-

sity of diffraction by a virus crystal (Section I,D) is at best about 1000 times less than that of a simple protein crystal

C Virus Crystals

1 Growing Crystals

To crystallize a virus all the particles must be of the same size and have the same surface structure There is nothing unusual about the ability of viruses to crystallize This is only an indication of the high degree of regularity and equivalence of the individual particles produced by a particular virus infection Crystallization occurs when large numbers of structurally equivalent particles (or molecules) are present in solution under conditions of limiting solubility Of all the possible interactions between particles there will be a certain set of contacts which will represent a potential energy minimum under the particular solvent conditions These minimum energy contacts will occur repeatedly, and *if the potential contact points are directed in three dimensions, the result* of this regular aggregation will be a three-dimensionally periodic crystal

The first practical problem in growing virus crystals is to obtain the virus particles in a purified, homogeneous form The conditions of purity required for crystallization are not very critical For example, contaminating low molecular weight material or particulate matter which might seriously influence chemical or physicochemical measurements is not likely to affect crystallization so long as the extraneous material does not randomly associate with the virus particles Extraneous material may be occluded in the crystals, but since such material will not be regularly arranged, it will not contribute to the diffraction pattern characteristic of the structurally regular virus particles, but will merely affect the background on the X-ray photograph Nonviral material which is attached to the virus in a regular way can crystallize with the virus and will contribute to the diffraction pattern Regular modifications of viruses are being continually sought for X-ray analysis, but so far, no heavy atom derivative of a spherical virus has been crystallized successfully

In some respects, the conditions of homogeneity required for crystallization are very stringent It is the surface of the virus particles that interact to form the crystal and these surfaces must, in crystallographic terms be identical A small amount of [illegible]

quite stable and this kind of denaturation is not commonly observed Since it is only the surface properties of a virus that affect its crystalliza-

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1 Growing Crystals

To crystallize a virus all the particles must be of the same size and have the same surface structure There is nothing unusual about the ability of viruses to crystallize This is only an indication of the high degree of regularity and equivalence of the individual particles produced by a particular virus infection Crystallization occurs when large numbers of structurally equivalent particles (or molecules) are present in solution under conditions of limiting solubility Of all the possible interactions between particles there will be a certain set of contacts which will represent a potential energy minimum under the particular solvent conditions These minimum energy contacts will occur repeatedly, and if the potential contact points are directed in three dimensions, the result of this regular aggregation will be a three-dimensionally periodic crystal

The first practical problem in growing virus crystals is to obtain the virus particles in a purified, homogeneous form The conditions of purity required for crystallization are not very critical For example, contaminating low molecular weight material or particulate matter which might seriously influence chemical or physicochemical measurements is not likely to affect crystallization so long as the extraneous material does not randomly associate with the virus particles Extraneous material may be occluded in the crystals, but since such material will not be regularly arranged, it will not contribute to the diffraction pattern characteristic of the structurally regular virus particles, but will merely affect the background on the X-ray photograph Nonviral material which is attached to the virus in a regular way can crystallize with the virus and will contribute to the diffraction pattern Regular modifications of viruses are being continually sought for X-ray analysis, but so far, no heavy atom derivative of a spherical virus has been crystallized successfully

In some respects, the conditions of homogeneity required for crystallization are very stringent It is the surface of the virus particles that interact to form the crystal and these surfaces must, in crystallographic terms, be identical A small amount of denaturation leading to randomization of the polypeptide configuration of the protein subunit could be sufficient to prevent crystallization Fortunately, many small viruses are quite stable and this kind of denaturation is not commonly observed Since it is only the surface properties of a virus that affect its crystalliza-

tion, the insides of the virus particles in a crystal need not all be the same A striking example of particles with inhomogeneous insides forming mixed crystals is provided by turnip yellow mosaic virus (TYMV) (Markham and Smith, 1949) and its associated RNA-free protein particle (see Section III,F) In this case it is necessary to conclude that the surface properties of the protein shell are unaffected by the presence or absence of the RNA core

Because of their structural properties, most spherical viruses can be crystallized reproducibly All the spherical viruses which have been crystallized appear to have a protein shell made up of a fixed number of structurally identical subunits Viruses in this category have a fixed size and shape and equivalent surface properties

The purification methods routinely employed for spherical viruses (see, for instance, Steere, 1959) will yield preparations that can be crystallized The detailed conditions for reproducibly growing crystals of size suitable for X-ray work have, however, only been worked out in the case of TYMV (Hindley and Klug, unpublished), using the type of procedure followed by protein crystallographers, and based mainly on the experience of the Cambridge school The general conditions are obvious. the virus should be in a state of limiting solubility and at a reasonable concentration Reasonable concentration in most cases is about 1%, but the optimum concentration should be determined for each particular virus and solvent

There are two methods of reducing the solubility which have been successfully applied in a number of cases The first is to add salt (generally ammonium sulfate) to the virus solution almost to the point where the virus is salted out Crystals will then generally begin to grow after a few days or a week It is helpful to allow very slow evaporation of water during this period For TYMV solutions at a concentration below ½% the optimum ammonium sulfate concentration at room temperature lies in a rather narrow range from 0 85 to 1 0 M Crystals may also be grown in the refrigerator at about 2°C, but the salt concentration necessary is about 0 15 M greater (Finch, unpublished) For some viruses, such as bushy stunt, which have a negative temperature-solubility coefficient, the salt can be added until the solution becomes turbid at room temperature (Bawden and Pirie, 1938b) When this solution is refrigerated it will clear and crystals will gradually grow over periods of weeks or months The second method of crystallization is to reduce the ionic strength of the solution at a pH near the isoelectric point of the virus Both types of methods have been applied to Coxsackie virus by Mattern (Mattern and du Buy, 1956), and, not surprisingly, yield two morphologically distinct crystal forms

For single crystal X-ray work the minimum practicable dimension for a crystal is about 100μ, although dimensions up to about 1 mm are preferable The size of the crystals is largely determined by the rate of crystallization in general, the slower the rate, the larger the crystals The principal factors which govern the rate of crystallization are: (1) virus concentration, (2) ionic strength and salt composition, and (3) pH. If enough purified virus is available, a series of tubes should be set up varying all three of these parameters in the range where crystallization is known to occur, in order to determine the optimum conditions

2 Crystal Mounting

Virus crystals contain a large amount of water and this water is an important part of the crystal Drying leads to disordering of the virus packing arrangement and also to some disordering of the particle structure To study the structure of the native virus particles it is therefore necessary to obtain diffraction patterns from the wet crystals Some useful information can, however, be obtained from dried crystals, particularly for comparison with the results from electron microscopy (Cowan and Hodgkin, 1951), but the amount of detail observable is quite limited

Crystals are mounted wet by sealing them in thin-wall glass capillaries with most of the mother liquor removed Some virus crystals are unstable at room temperature and in some cases the high alkali content of the soft glass capillaries usually used has a deteriorative effect Crystals grown from low ionic strength solutions are the most sensitive in this respect. Finch and Klug (1959) found that poliovirus crystals, which are unstable under the conditions used for bushy stunt and turnip yellow mosaic virus crystals, could be stabilized by mounting in quartz capillaries and keeping them at a temperature of about 5°C Not only was deterioration prevented but the diffraction patterns obtained in this way show more detail than those obtained from any other crystalline virus In spite of the difficulties of specimen refrigeration during the long X-ray exposures, it would seem advisable to adopt this procedure for most single crystal virus studies

3 The Forces between Virus Particles

In crystals of the small viruses each particle is either in contact, or very nearly so, with its nearest neighbors The values of the interparticle distance in crystals are always greater than the diameter obtained by low-angle X-ray scattering from solutions (Table III), but the latter is only a spherically averaged mean value For a model such as that shown in Fig 13 the maximum diameter is about 10% larger than

the mean diameter and could be greater for more asymmetrically shaped subunits. It is therefore not necessary to postulate any layers of free water, at least none greater than a few molecules thick, between particles in a crystal (Klug *et al.*, 1957b). Virus crystals of this kind therefore resemble crystals of the globular proteins (Crick and Kendrew, 1957) in that they are held together by local contacts. The particular lattice adopted does depend, of course, on the over-all shape of the particle, but it also depends on the salt concentration and the pH. The forces which determine the crystal structure are noncovalent interactions such as hydrogen bonding, hydrophobic bonding, and salt linkages, forces which are of course just those which determine protein chain folding (Kauzmann, 1959).

When the virus particles are very large (~1000 A), however, long-range forces come into play (Bernal, 1946, 1959; Oster, 1950, 1957). These are well known from the phenomena of colloid physics, and, the most reliable information on them has come from Bernal and Fankuchen's classic study (1941) on TMV gels. Indeed, because of its uniformity in size and shape, TMV has proved an ideal model for the study of long-range forces as for a host of other biophysical studies. The separation of TMV solutions above a critical concentration into an ordered bottom layer and isotropic top layer (Bawden and Pirie, 1937a) is largely a thermodynamic consequence of the asymmetric particle shape (Onsager, 1949) but also depends on the long-range forces. Under certain conditions three-dimensionally periodic ordering of TMV can occur (p. 242).

Crystals of Tipula iridescent virus (TIV) (Williams and Smith, 1957; Smith and Williams, 1958) provide an interesting example of the long-range ordering of isodimensional colloid particles (Klug *et al.*, 1959). These are true crystals which have a unit cell so large that they diffract light just as ordinary crystals diffract X-rays, so that a crystallographic study is possible using visible light (Fig. 10). Klug *et al.* (1959) showed that the virus particles are packed in a face-centered cubic array, with an interparticle spacing of 2500 ± 30 A, a distance nearly twice the diameter of the frozen-dried particle. Even after allowing for the hydration of the particles in the crystal, they still cannot be in contact, but are separated by large distances of water of the order of 500 A. The macroscopic properties of these crystals are also consistent with the view that long-range forces are operative. The crystals are extremely fragile, they cannot be grown by simple concentration or precipitation, and are destroyed by the presence of small amounts of salt. Since TIV is obtainable in fairly large quantities and is readily purified, and, moreover, since the virus particles are both large and remarkably uniform, it seems



FIG 10 (a) Copies of a color photograph of a cell containing crystals of Tipula iridescent virus taken by back reflection in white light (Klug *et al*, 1959) The crystals appear as small black patches except where they happen to be set in the right orientation to give Bragg reflections of the incident light These flash brilliant green or blue, and appear white in this print

(b) The same, but with the cell rotated to a slightly different position (as shown by the position of the cross wires) Different crystals are now in the correct setting to give Bragg reflections

that this virus may provide the ideal material for quantitative studies on a colloidal system consisting of isometric particles, just as TMV is an ideal model for an anisometric particle

Some information on the interaction of virus particles in solution can be obtained by studying the interparticle interference effects found in the X-ray scattering by fairly concentrated virus solutions A beginning in this direction has been made for a spherical virus by Schmidt *et al.* (1954) and for TMV by Caspar (1955) In neither case do the virus particles behave simply as a "gas" of hard spheres or cylinders, presumably because of the electrostatic repulsive forces between them

D *Spherical Viruses and Cubic Symmetry*

Symmetry is a mathematically precise concept and the mathematics of symmetry is the foundation of crystallography The nonmathematical reader who is not concerned with these exact concepts as they relate to virus crystal and particle structure might not be interested in the

following two sections However, the biochemist or electron microscopist who is investigating virus substructure canont ignore the restrictions on virus structure imposed by symmetry. Determination of the particle symmetry is only the first step in the X-ray analysis of virus substructure The relation between the crystallographer's subunit and that detected by chemical methods or that seen in the electron microscope need not always be as obvious as is the case for TMV. The problem of the relation of virus symmetry to virus morphology is considered in Section III,D,3, below

1 The Symmetry of Spherical Virus Particles

We have already discussed how regular packing of subunits leads to a symmetrical structure (Section I,C). In the helix, each unit is related to the next by a rotation plus a translation Since the angle between subunits along a helix can take any value, there is no geometrical restriction on the number that can be placed in one turn, nor need this number even be integral Furthermore, the length of a helical structure such as TMV is not determined by its symmetry since the structure can repeat indefinitely along a line (the helix axis) A helical arrangement of subunits is therefore theoretically infinite in extent, and we have seen that the length of the TMV helix is determined by the RNA

That only a limited number of kinds of symmetry is possible for a spherical virus particle was first clearly pointed out by Crick and Watson (1956) For a particle of finite extent, built up of subunits regularly packed about a central point rather than about a line, the only symmetry elements permissible are rotation axes, if the subunits are optically active molecules of one hand only The number of asymmetric units must be a certain definite integer depending on the particular combination of rotation axes present

The simplest arrangement would be that due to a single rotation axis which results if an integral number of subunits are regularly arranged around a circle, if the number is n, the axis is called n-fold This sort of aggregate would, however, only appear spherical if the subunits had the approximate shape of the sectors of an orange Symmetry of a higher order results when there are a number (or "group") of rotation axes intersecting at a point The number of ways in which various n-fold axes can be combined are very limited and the arrangements possible are known as *point groups* Twofold axes can be combined at right angles with any single n-fold axis, and although it is possible that some virus particles may have symmetry of this type, this will not in general lead to a particle that appears spherical or even isodimensional The only other possible combinations of rotation axes are the three types of cubic symmetry (cubic point groups) which each have at least one

set of four 3-fold axes arranged as the four axes from the center to the vertexes of a tetrahedron or, equivalently, as the four body diagonals of a cube The number and relative arrangements of rotation axes for these three types of cubic symmetry can be represented by the tetrahedron, the cube, and the icosahedron (Fig 11), the number of asym-

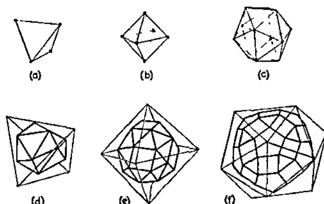


Fig 11 Top Regular (Platonic) polyhedra, in which the respective arrangements of rotation axes represent the three types of cubic symmetry possible for a spherical virus The solids chosen each have a set of faces perpendicular to their 3-fold axes A cube and a regular pentagonal dodecahedron could also have been used to represent (b) and (c), respectively

(a) Tetrahedral symmetry four 3-fold axes (through the vertexes) and three 2-fold (through the mid-points of opposite edges)

(b) Octahedral symmetry three 4-fold axes (through opposite vertexes), four 3-fold axes (through centers of opposite faces), and six 2-fold axes (through mid-points of opposite edges)

(c) Icosahedral symmetry six 5-fold axes (through opposite vertexes), ten 3-fold axes (through the centers of opposite faces), fifteen 2-fold axes (through the mid-points of opposite edges)

Bottom Polyhedra in which the arrangements of vertexes represent more general realizations of the symmetry type shown above The number of vertexes in (d), (e), and (f) is 12, 24, and 60, respectively, equal to the number of asymmetric units required by the symmetry Thus, in each case, identical subunits placed (in suitable orientations) at the vertexes would all be equivalent, no matter what their shape or structure

(d) A general polyhedron with tetrahedral symmetry

(e) Snub cube

(f) Small rhombicosadodecahedron

(e) and (f) are rather special polyhedra, because each has all its edges equal (Archimedean polyhedra) this condition is not required by the symmetry They have been chosen here because they are well known (Cundy and Rollett, 1951) All the edges in (d) cannot be made equal since a regular icosahedron would result, and the symmetry would be increased

metric units required by each of these three point groups is, respectively, 12, 24, and 60 The pertinent information is summarized in Table IV Subunits arranged with cubic symmetry will all be equidistant from the center of the point group, and will thus form an approximately spherical or polyhedral shell

TABLE IV
THE THREE POSSIBLE (NONENANTIOMORPHOUS) CUBIC POINT GROUPS FOR A SPHERICAL VIRUS[a, b]

Crystallographic description[c]	No and type of rotation axes present	No of asymmetric units	Platonic solid with these symmetry elements
23	3 2-fold	12	Tetrahedron
	4 3-fold		
432	6 2-fold		
	4 3-fold	24	Cube
	3 4-fold		Octahedron
532	15 2-fold		
	10 3-fold	60	Dodecahedron
	6 5-fold		Icosahedron

NOTE The number of subunits will be the same as, or a multiple of, the number of asymmetric units

[a] See Fig 11

[b] From Crick and Watson (1956)

[c] *Pronounced two-three, four-three-two, and five-three-two* The notation refers to the symmetry axes present

The relevance of these considerations to the structure of spherical viruses was discussed by Crick and Watson (1956), who predicted that the small spherical viruses would turn out to have one or other of the three types of cubic symmetry As we shall see later, the spherical viruses so far investigated have all turned out to have only icosahedral symmetry

2 Particle and Lattice Symmetry

A crystal lattice is characterized not only by the size and shape of the unit cell but also by the symmetry elements which are present This assembly of symmetry elements is called the *space group* of the crystal, since the elements must repeat throughout space from cell to cell, in contrast to the case of a point group, where the symmetry elements are clustered around one point. Every lattice point of a crystal will, however, constitute just such a center of a point group, the symmetry of which must be related in a very direct manner to the symmetry of the

space group Thus we can place a tetrahedron or octahedron at every lattice point of a cubic point lattice, and still retain the cubic symmetry throughout space, by orientating the polyhedron so that the directions of its four 3-fold axes coincide with the four body-diagonals More generally, any set of objects with tetrahedral or octahedral cubic point-group symmetry (see Table IV) can be arranged in various types of cubic lattice in this way It is a little more difficult to see that the same can be done with an icosahedron or a more general object with icosahedral symmetry The solution is illustrated in Fig 12 This is the

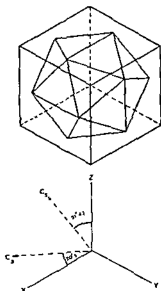


Fig 12 The icosahedron inscribed in the cube (Euclid) Note that the icosahedron can be rotated through 90° with respect to any of the cube axes to another equivalent position

classical construction of the icosahedron inscribed in the cube Of the ten 3-fold axes of the icosahedron, four lie along the 3-fold axes of the cube and may be said to build the crystal The remaining six lie in noncrystallographic directions, as do all the 5-fold axes and all but those three 2-fold axes which lie parallel to the cube edges

It is natural for particles possessing cubic point-group symmetry to crystallize in a lattice with cubic symmetry If it is known that the crystal lattice possesses true cubic symmetry, and that there is only one

particle per lattice point, then it follows that the virus particle itself must have at least the lowest form of cubic symmetry (tetrahedral) and so be made up of at least 12 subunits This argument (Hodgkin, 1949, Low, 1953) was first used to point to the possible existence of subunits in spherical viruses The particle symmetry may be still higher (i e, octahedral or icosahedral), but it cannot be lower. The choice between tetrahedral and octahedral symmetry can usually be made by a formal crystallographic study of the crystal symmetry.

The detection of icosahedral symmetry is less straightforward, since the additional rotational axes characterizing it are noncrystallographic or irrational This is related to the fact that no space group or crystal lattice can possess 5-fold axes (To illustrate the point, consider the impossibility of tiling a floor with tiles in the shape of regular pentagons) It was pointed out by Caspar (1956b) that the presence of these noncrystallographic axes could be detected by considering the distribution of intensity in the diffraction pattern of a crystal. It can be shown that the intensities of reflections lying in or near the directions of these symmetry axes would tend to be greater than the average, and that these directions would therefore stand out as "spikes" on the X-ray photographs The theory of diffraction by the cubic point groups can be worked out in a form analogous to that for a helix (Klug, in preparation)

It should, however, be stressed that even though the virus particles might possess cubic symmetry, it might well happen that they crystallize in a lattice of lower symmetry Thus poliovirus, the particles of which have icosahedral symmetry, crystallize from phosphate-saline mother liquor in an orthorhombic lattice, in which the only symmetry elements the lattice has in common with the particle are the three 2-fold axes along the cell edges As we shall see, however, the arrangement is still pseudo-body-centered cubic

A fairly wide variety of crystal forms might be expected for roughly spherical particles without strong directional interactions, the particular lattice adopted depending on the solvent conditions Turnip yellow mosaic virus, for instance, crystallizes in a cubic form from ammonium sulfate and in a noncubic form from alcohol, crystallization has also been effected by salts such as sodium thiosulfate, magnesium sulfate, and sodium chloride (Markham and Smith, 1949) Coxsackie virus crystals (Mattern and du Buy, 1956) grow in an orthorhombic form from ammonium acetate and in an apparently cubic form from normal saline

3. *Symmetry and Morphology*

The elements of substructure deduced in a virus particle will depend on the method used for revealing them. For example, these may be the

individual protein molecules which make up the shell, the asymmetric unit which is the smallest crystallographic subdivision, or the knobs seen in high-resolution electron micrographs. These chemical, crystallographic, and morphological subunits must necessarily be related but need not be the same As far as the actual assembly of the virus is concerned, the most important subdivision of the particle may be the groups of molecules which aggregate to form the coat for the nucleic acid core It is conceivable that this building unit is not identical to any of the three identifiable types of subunits just listed, but of course it must be intimately related to them Determination of the size and number of chemical, crystallographic, or morphological subunits in a virus does not explain how these units come together to form the shell What we ultimately need to establish is the regular contact pattern between individual protein molecules The regular shell is a consequence of this contact pattern, that is, the shell is assembled from the protein molecules arranged in definite groups Since we do not yet have techniques for following the process of virus particle assembly, we must deduce the construction principles from the symmetry and morphology of the finished product In the case of TMV some of these principles have been elucidated (Section II,D), but with our limited knowledge of spherical virus structure we can, at best, only suggest some tentative models

We will consider some of these models to indicate the nature of the structural problem before discussing the experimental results As we shall see, all the small viruses so far studied by X-ray methods appear to have icosahedral symmetry which implies they consist of 60 asymmetric units How can 60 equivalent units be arranged with icosahedral symmetry? There are actually an infinite number of slightly different models, but all can be visualized as consisting of 12 identical groups of 5 units equivalently related by the 5-fold axes of the icosahedral point group If we make the restriction that the structure be as compact as possible, which implies that it has the maximum stabilization from intersubunit contacts, and moreover assume that the subunits are approximately spherical in shape, the model shown in Fig 13 results This is not an unreasonable model for an icosahedral virus, but if the asymmetric units are not spherical in shape a particle of very different appearance could result For example, if the spheres in Fig 13 were transformed into ellipsoids extending out from the center so that the ends of groups of 5 come together compactly at a larger radius, the structure would appear, at lower resolution, to consist of only 12 knobs On the other hand, if ellipsoids are clustered in groups of 3 about the

particle per lattice point, then it follows that the virus particle itself must have at least the lowest form of cubic symmetry (tetrahedral) and so be made up of at least 12 subunits This argument (Hodgkin, 1949, Low, 1953) was first used to point to the possible existence of subunits in spherical viruses The particle symmetry may be still higher (i e, octahedral or icosahedral), but it cannot be lower. The choice between tetrahedral and octahedral symmetry can usually be made by a formal crystallographic study of the crystal symmetry.

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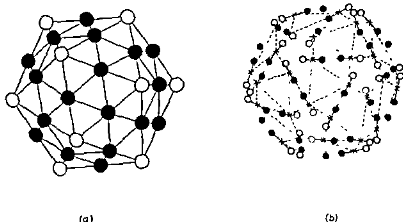


FIG 14 An example illustrating the relation between the morphological subunit and asymmetric unit

(a) An arrangement of 42 units consisting of 12 (white) placed at the vertexes of a regular icosahedron and 30 (black) at the mid-points of the edges This arrangement would have strict icosahedral symmetry only if either (i) each unit were a structureless sphere or point or (ii) each unit were subdivided as in (b), i e, each white unit into 5 subunits, and each black into 2, making 60 each of black (12 × 5) and white (30 × 2) subunits

(b) A subdivision of (a) resulting in an arrangement possessing strict icosahedral symmetry and consisting of 120 subunits of two kinds (black and white) The two kinds of subunit need not be the same chemically, but even if identical, they are still differently situated in the structure (i e, they are not structurally equivalent)

The appearance of the whole assembly can vary widely, depending on the relative separation of the two parts (black and white) of the asymmetric unit and on their size and shape It could even, at low resolution, resemble Fig 14(a) or some other gross arrangement with a number of knobs equal to $(60/m + 60/n)$ where m and n are any of the integers 5, 3, or 2

If we know the number of chemical units in the asymmetric unit and the number of morphological units seen in the electron microscope, the probable contact arrangement of the protein molecules can be deduced from the symmetry The most probable arrangement is that in which each protein molecule in the virus particle regularly forms the maximum number of stabilizing bonds with its neighbors The critical test of any model will be to show by calculation that it agrees in detail with at least the low-angle part of X-ray diagrams As yet no such realistic model has been constructed for any spherical virus, but with the recent advances in the electron microscopy of small viruses (Section III,I,2) this may soon be possible

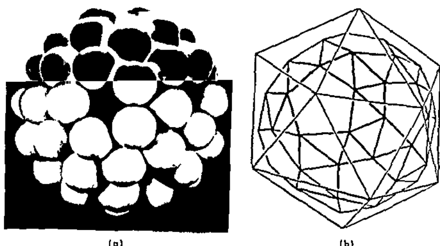


FIG. 13. (a) Schematic model illustrating one way in which 60 subunits may be packed according to icosahedral symmetry to form the protein shell of a spherical virus. The subunits are arranged at the vertexes of a snub dodecahedron (b), which has the same rotational symmetry as the icosahedron in which it is shown inscribed.

Ping-pong balls have been used for convenience in building the model, but do not represent the actual shape of protein subunits. Whatever shape is used in a model of this kind, the geometrical relation between each subunit and its neighbors remains the same for all subunits. Each such structural subunit may be further divided into two or more nonequivalent units. This is illustrated in Fig. 14(b).

3-fold axes or in groups of 2 about the 2-fold axes, there would be 20 or 30 knobs, respectively.

Other numbers of morphological bumps on the particle are possible if the asymmetric subunit is itself divided into an integral number of smaller chemical subunits. These protein molecules need not be all identical (nor need they be different), since the icosahedral symmetry only requires that groups of 60 be equivalent. Each group of 60 can cluster about the 5-, 3-, or 2-fold axes or may occur in general positions. The number of morphological units can be found by adding some combination of the numbers 12, 20, 30, and 60 or a multiple of 60 appropriate for the particular clustering arrangement. Each set of equivalent bumps of a particular type will consist of 60 (or a multiple of 60) chemical units, and there cannot be more sets than there are chemical units in one asymmetric unit. This may all seem rather complicated, but any particular model of this type is easy to construct. A diagram representing one of the many models of this type, consisting of 120 units arranged in 42 clusters, is shown in Fig. 14.

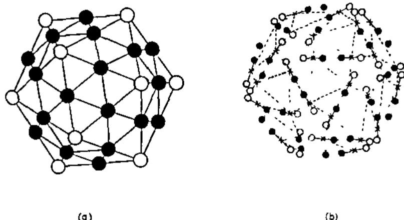


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E Tomato Bushy Stunt Virus (BSV)

1 Crystal Structure

This virus was not only the first spherical virus to be crystallized but also the first on which definite evidence for substructure was obtained The early X-ray studies (Carlisle and Dornberger, 1948) on crystals grown from ammonium sulfate showed that the unit cell was cubic in shape with a cell side of 386 Å, but this did not establish definitely that its symmetry was cubic Caspar (1956b) obtained X-ray precession photographs that proved the symmetry to be cubic. The crystal lattice is *body-centered cubic*, that is, the unit cell contains virus particles at the corners of the unit cube and one in the center. Each particle is thus surrounded by eight nearest neighbors

The interparticle distance between nearest neighbors in the wet crystals is 334 Å but the mean diameter of the hydrated particle measured by low-angle scattering is only 309 Å There is evidence, however, that the BSV particles must be in contact in the crystal lattice. The effect of change of temperature and salt concentration on the crystallization, considered in terms of the expected behavior of hydrophobic bonds as described by Kauzmann (1959), indicates that such bonds must occur between virus particles A hydrophobic bond can only form if a nonpolar part of one particle is in close contact with a nonpolar part of another particle Thus the maximum diameter of BSV must be at least 334 Å The observed difference between the maximum and mean diameter is just such as might be expected for a particle with the type of substructure indicated in Fig 13

The value of the particle diameter, 300 ± 10 Å, obtained by electron microscopy of frozen-dried preparations (Williams, 1953, Kaesberg, 1959), is also less than the interparticle distance in the wet crystals This is found to be the case in other comparisons of this kind (Table III). X-ray measurements on dried crystals are difficult to make because the diffraction pattern fades out very sharply, but observations on BSV crystals, strongly dried over P_2O_5 (Carlisle and Dornberger, 1948) give an interparticle distance of only 267 Å. This result indicates that the particle itself is shrinking on drying Since the virus particle may be regarded as a kind of miniature protein crystal, this might be expected.

2 Substructure of the Virus Particle

In a simple body-centered cubic lattice all the particles have the same orientation so that the diffraction pattern of the BSV crystal relates

directly to the diffraction from a single virus particle (In crystallographic jargon, the scattering by a single particle is "sampled" at the points of the reciprocal lattice)

The symmetry of the crystal lattice is tetrahedral which implies that the individual virus particles have at least this symmetry and must therefore be made up of 12, or a multiple of 12, identical subunits The virus point-group symmetry may still be higher (but not lower) than that determined by the crystal, and indeed Caspar (1956b) has shown this to be the case He observed that the distribution of the strong reflections in the diffraction pattern was highly suggestive of the icosahedral point group There is a concentration of reflections of high intensity in directions bearing the same relation to each other as the symmetry axes of this point group (Fig 15a) This indicates that the virus particle has icosahedral symmetry, which implies that it is built up of 60 structurally equivalent asymmetric units

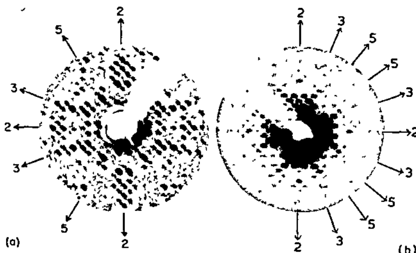


FIG 15 X-ray diffraction diagrams (precession photographs) of single crystals of (a) tomato bushy stunt virus (Caspar, 1956b), and (b) turnip yellow mosaic virus (Klug *et al*, 1957b, Finch, 1959), taken in each case with the X-ray beam parallel to the edge of the cubic unit cell Only the central region of each pattern is shown

Many such photographs must be taken along different crystal directions in order to record the complete three-dimensional diffraction pattern of a crystal

The directions of the 2-fold, 3-fold, and 5-fold rotation axes corresponding to icosahedral symmetry, which lie in the plane of the photograph, are marked by arrows The set of 2-fold axes is parallel to the cube edges of the crystal, but the

E. Tomato Bushy Stunt Virus (BSV)

1. Crystal Structure

This virus was not only the first spherical virus to be crystallized but also the first on which definite evidence for substructure was obtained. The early X-ray studies (Carlisle and Dornberger, 1948) on crystals grown from ammonium sulfate showed that the unit cell was cubic in shape with a cell side of 386 Å, but this did not establish definitely that its symmetry was cubic. Caspar (1956b) obtained X-ray precession photographs that proved the symmetry to be cubic. The crystal lattice is body-centered cubic, that is, the unit cell contains virus particles at the corners of the unit cube and one in the center. Each particle is thus surrounded by eight nearest neighbors.

The interparticle distance between nearest neighbors in the wet crystals is 334 Å but the mean diameter of the hydrated particle measured by low-angle scattering is only 309 Å. There is evidence, however, that the BSV particles must be in contact in the crystal lattice. The effect of change of temperature and salt concentration on the crystallization, considered in terms of the expected behavior of hydrophobic bonds as described by Kauzmann (1959), indicates that such bonds must occur between virus particles. A hydrophobic bond can only form if a nonpolar part of one particle is in close contact with a nonpolar part of another particle. Thus the maximum diameter of BSV must be at least 334 Å. The observed difference between the maximum and mean diameter is just such as might be expected for a particle with the type of substructure indicated in Fig. 13.

The value of the particle diameter, 300 ± 10 Å, obtained by electron microscopy of frozen-dried preparations (Williams, 1953; Kaesberg, 1959), is also less than the interparticle distance in the wet crystals. This is found to be the case in other comparisons of this kind (Table III). X-ray measurements on dried crystals are difficult to make because the diffraction pattern fades out very sharply, but observations on BSV crystals, strongly dried over P_2O_5 (Carlisle and Dornberger, 1948) give an interparticle distance of only 267 Å. This result indicates that the particle itself is shrinking on drying. Since the virus particle may be regarded as a kind of miniature protein crystal, this might be expected.

2. Substructure of the Virus Particle

In a simple body-centered cubic lattice all the particles have the same orientation so that the diffraction pattern of the BSV crystal relates

Hersh and Schachman (1958) have observed in the ultracentrifuge a protein unit of weight about 60,000—a value in agreement with the end group results The protein units observed, although presumably no longer native, can be considered as molecular subunits of the virus which, before degradation, were held together by secondary forces to form the organized protein framework characteristic of the virus particle The subunit detected by chemical and physicochemical methods is about half the size of the asymmetric unit deduced from the X-ray analysis It thus appears possible that the particle of BSV is built up of 120 protein subunits, or 60 pairs, arranged in some way so that the whole has icosahedral symmetry These protein molecules may all be the same, or may be of two or more different types

Very little is known about the structure of the RNA of the virus Recently, the isolation of an infectious RNA preparation from purified BSV has been reported (Rushizky and Knight, 1959) The preparation contained a small amount of proteinaceous material, but it is not known whether this is indispensable for infectivity It also appears from preliminary experiments (H K Schachman, personal communication) that this infectious RNA is of high molecular weight, approaching the total RNA content of the virus This is analogous to the results with TMV, and the infectious unit may again turn out to be the whole chain of RNA in the virus

F Turnip Yellow Mosaic Virus (TYMV)

This virus is of considerable interest because the infective virus is accompanied in the plant by a physically similar protein component (the "top component") (Markham and Smith, 1949, Markham, 1951) The protein particles are noninfective and contain no RNA This gave the first indication that it was the RNA of a plant virus which might be responsible for infectivity The virus has a molecular weight of 5×10^6, contains about 40% RNA, and has a spherically averaged diameter of 280 Å as measured by low-angle X-ray scattering The protein particle is also nearly spherical, having about the same external diameter as the complete virus, and Markham (1951) suggested that it had the approximate form of a hollow spherical shell This was confirmed by Schmidt *et al* (1954) by means of low-angle X-ray scattering on solutions

1 Crystal Structure

Both the virus and the nucleic acid-free top component can be crystallized from ammonium sulfate solution (Markham and Smith, 1949) and will indeed form mixed crystals Bernal and Carlisle (1948) made the first X-ray study of the virus by means of "powder" photographs and,

It is not possible to prove from the X-ray photographs so far obtained, which were limited to spacings greater than about 20 Å, that the virus particle possesses strict icosahedral symmetry. There may be some arrangement of subunits simulating this symmetry. This could come about, for instance, if there were 60 subunits, not all identical, but located at a set of points having icosahedral symmetry. Pseudo-532 symmetry of this kind could also arise if there were 12 subunits of protein or nucleic acid arranged at the 12 vertexes of an icosahedron. Only if each of these subunits were made up in turn of 5 sub-subunits could the whole arrangement have strict icosahedral symmetry, and here we would be back again to 60 (= 12 × 5) equivalent subunits. This point is illustrated by a more complex example in Fig. 14. It is also not possible to tell in a simple manner whether the 532 symmetry is only partially present in the complete virus particle. It might be possessed by only one of the two components present, namely, the protein or the nucleic acid.

Nevertheless it seems very likely that the protein coat of BSV consists of 60 asymmetric units, since we do have a very similar case—that of TYMV—where it has been possible to show (Section III,F,3) that the protein component of the virus does have icosahedral symmetry.

3. *Chemical Evidence on Substructure*

The molecular weight of BSV is close to 9×10^6 (Williams and Backus, 1949; Cheng and Schachman, unpublished: quoted in Hersh and Schachman, 1958), of which about 17% is ribonucleic acid (de Fremery and Knight, 1955). The molecular weight of each of the 60 structurally equivalent protein subunits is therefore 125,000. The point-group symmetry does not preclude the possibility of each crystallographic asymmetric unit being subdivided further into a number of chemically identical protein molecules. Thus, 125,000 is a maximum value for the size of the protein subunit. Caspar (1956b) pointed out that the amino acid analysis of BSV protein (de Fremery and Knight, 1955) indicates a minimum molecular weight of 25,000–28,000 for the chemical unit.

The end-group analyses made by Niu *et al.* (1958) indicate that the chemical subunit has a molecular weight of about 57,000, i.e., about twice the above value. Using sodium dodecyl sulfate to degrade the virus,

other two sets do not lie along crystallographic axes. There is a concentration of strong reflections ("spikes") in these directions. There are twice as many such spikes in the TYMV pattern as in the BSV, because the virus particles in the crystal of TYMV are arranged in two different orientations (rotated 90° to each other about the cube edge of the crystal) (Fig. 16). In the BSV crystal, all the virus particles have the same orientation.

2 The Symmetry of the Virus Particle

The crystal structure just described requires, just as does that of BSV, that the virus particle have tetrahedral (23) symmetry We have already discussed the additional 5-fold symmetry in BSV, which was recognized by the concentration of strong reflections in the directions of the symmetry axes of the point group 532 The precession photographs of TYMV also show strong indications of icosahedral (532) symmetry, but here there are twice as many of these spikes of strong reflections (Fig 15b) These spikes can be resolved into two identical sets rotated 90° to each other about the cube edge of the crystal, corresponding to the two different particle orientations present in the crystal Moreover, the relative directions of the spikes, so resolved, are identical with those of BSV, thus it can be concluded that TYMV, like BSV, has icosahedral symmetry properties

The larger unit cell of TYMV gives rise to more reflections than does a BSV crystal, and the higher symmetry allows the possibility of determining directly whether the 532 symmetry indicated by the spikes is fully present or not Members of a certain class of reflections are observed, which, if the particle had true 532 symmetry, would be absent It was therefore concluded that the TYMV particle had 23 symmetry, with additional pseudo-symmetry of the point group 532 We have already discussed (p 302) a number of ways in which this circumstance might come about An arrangement merely simulating 532 symmetry can, however, be almost certainly ruled out, even at this stage of the analysis, because the spikes of intensity extend clearly as far out as spacings of 10 Å, and some strong reflections lie very close to the 5-fold symmetry axes in the 5 Å region It is highly unlikely that this could arise from an arrangement of subunits which did not have true 532 symmetry in one of the two components Thus the TYMV particle presents a case of partial rather than merely pseudo-532 symmetry The existence of the top component associated with the virus and the fact that it crystallizes in the same form as the virus provides an exceptional opportunity for studying the substructure of both the protein and the RNA in a spherical virus, on the lines of what has already been done for TMV and repolymerized A-protein

3 The Symmetry and Structure of the Top Component

The question of whether or not the virus protein has full 532 symmetry, and therefore consists of 60 asymmetric units, can best be answered by studying single crystals of the top component Crystals large enough for this purpose have not been prepared until recently, the first

when larger crystals became available, they obtained "still" photographs of single crystals (Bernal and Carlisle, 1951) From these X-ray studies it was shown that the unit cells of both the virus and protein are cubic, and of sides 703 and 715 Å, respectively, but the small difference between these values is not much greater than the experimental error.

Because of the limited data available, the crystal structure proposed by Bernal and Carlisle was not certain The problem was taken up again by Klug *et al* (1957a,b), who obtained precession photographs from which the structure could be solved The centers of the virus particles fall on a simple body-centered cubic lattice with a cube edge of 350 Å, but the particles have either of two orientations which alternate in a regular manner (Fig 16) This means that the true unit cell is

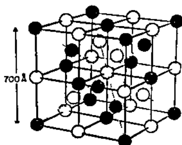


FIG 16 The unit cell of turnip yellow mosaic virus (in ammonium sulfate) The black and white spheres represent identical virus particles set in two different orientations Note that the centers of the particles fall on a body-centered cubic lattice

larger than the simple one corresponding to the virus centers At low resolution, when the two orientations of the virus particles are indistinguishable from each other, the crystal structure is a simple body-centered cubic one, with an interparticle distance of 304 Å, and therefore similar to that of bushy stunt virus (BSV) Formally, the structure may be thought of as made up of two interpenetrating diamond-type lattice.

The very large unit cell of the TYMV crystal increases by almost an order of magnitude the experimental difficulties over those of a crystal like BSV, which are already severe However, the additional complexity of the TYMV lattice has proved an advantage in making a rigorous analysis of the symmetry of the virus particle, although without the evidence already obtained from BSV this symmetry might not have been recognized

2. The Symmetry of the Virus Particle

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X-ray investigations (Klug *et al*, 1957b) were made on a "powder," or sludge of small wet crystals. The work was essentially an extension to much larger angles of the measurements made by Schmidt *et al.* (1954) on the protein particles in solution. It was confirmed that the particles, at low resolution, appeared to be hollow spheres with spherically averaged internal and external diameters of 210 and 280 Å Moreover, at higher angles of scattering a marked modulation in the intensity pattern showed that the shell is not homogeneous, and indicated a predominant spacing of about 60 Å in the protein particle

Recently Klug and Finch (1960, Finch, 1959) have obtained X-ray precession photographs of a small, single crystal of top component from a batch prepared by Markham The diffraction from a crystal of top component is much weaker than that from the virus crystal, weak as that is already, so that only a very limited part of the pattern can be recorded There is enough information, however, to establish that the protein particle has 532 symmetry "Spikes" of strong intensity are present, moreover, they are enhanced with respect to the corresponding spikes in the diffraction pattern of the virus crystal The protein is therefore the seat of the icosahedral symmetry in the virus particle and thus it is built up of 60 asymmetric units

The 60 Å spacing observed in the protein particle is consistent with the arrangement of 60 globular units with icosahedral symmetry on the surface of a sphere Accordingly such a model (Fig 13) was put forward (Klug *et al*, 1957b) It was pointed out that this model is schematic, unlike that of TMV (Fig 1) which is reasonably realistic The model shown in Fig 13 is only the simplest one providing an explanation of the X-ray results The modifications of the structure discussed in Section III,D,3 could lead to a very different morphological appearance High-resolution electron micrographs of TYMV (Huxley and Zubay, 1960, Nixon and Gibbs, 1960) provide the additional information necessary for the construction of a more realistic model (Klug and Finch, 1960) It now appears that the 60 Å spacing is characteristic of the distance between the crevices in the shell rather than between protuberances (see p 314)

4 Remarks on the Structure of RNA in the Virus

The X-ray precession photographs from the protein particle and the virus are generally very much alike As the external shapes and sizes are also very similar, the virus protein apparently keeps the same or a similar configuration whether or not the nucleic acid is present. The specific differences between the photographs of protein and of virus

must be due to the RNA The fact that these differences are not spherically symmetric suggests that the RNA has a definite structure

Some light may be shed on this question by comparing the wide-angle powder photographs of virus and top component The difference between the two scattering curves, which represents the contribution of the virus RNA, bears little resemblance to that of isolated RNA (Franklin *et al*, 1958). Thus, much of the RNA in the virus particle appears to have a structure different from that of isolated RNA and presumably one imposed upon it by the virus protein

If the protein has strict 532 symmetry, the 23 symmetry of the virus particle as a whole (which certainly extends to a resolution of at least 10 Å) must include the RNA This is not only strongly implies that the RNA has a definite structure, but also suggests that it might have 12 (geometrical) subunits This does not mean that chemically the RNA cannot be a single chain, for in this case the "subunits" would refer to the mode of winding of the chain However, we cannot yet be definite on the number of structural units of RNA, since the tetrahedral symmetry suggested for the RNA refers to the average throughout the crystal This ambiguity can, however, be settled eventually when more data are available

Determination of the symmetry of the RNA does not establish how the polynucleotide chain is packed within the framework of protein subunits One cannot simply make a list of the *a priori* geometrical paths possible, as could be done in the case of TMV (Section II,E,*1*), where the helical symmetry lends itself naturally to the accommodation of a chain molecule

The amino acid analysis of TYMV (Markham, 1959) shows that there are not enough basic residues present (as there are in TMV) to neutralize the charge on the RNA In any case, since the RNA is located mainly within the protein shell, all of it presumably cannot be in contact with protein It is probably coiled inside the virus, and Markham (1959) suggests that it is probably cross-linked by means of polyvalent bases

5 *Chemical Evidence on Substructure*

The state of the investigations in this field rather resembles the stage reached in the case of BSV The amino acid analyses of TYMV (Markham, 1959) give by calculation a minimum subunit size of about 20,000, and the preliminary end-group analysis (J I Harris, personal communication) indicates that this chemical subunit is indeed of this size On the basis of the X-ray analysis, the structural subunit has a molecular

weight of 50,000, so that it therefore contains either 2 or 3 chemical units

The degradation of the virus has been studied by Lyttleton and Matthews (1958), who found that the RNA could be released by heating under rather mild conditions, without any obvious gross breakdown of the protein shell Kaper and Steere (1959) have reported an alkaline degradation method by means of which small soluble protein fragments could be obtained It is not yet clear what the relation is of these fragments to the protein subunits and whether they are still native Kaper and Steere have also isolated an infectious RNA fraction from TYMV. Attempts to reconstitute whole virus from the protein fragments and this TYMV-RNA have so far failed Very recently, Kaper (1960) has presented evidence on the sequence of events during the alkaline degradation His experiments suggest that first intact protein shells, resembling top component, are formed and that these then dissociate into particles of smaller size It would be most interesting if "artificial" top component could be produced in this way, by careful control of the conditions *

G Poliovirus

Poliovirus was the first animal virus to be crystallized (Schaffer and Schwerdt, 1955), and the event therefore aroused a great deal of interest From the X-ray results, it is clear that there are no structural grounds for distinguishing the smaller animal viruses from the plant viruses The principal reason why few crystals of animal viruses have been reported is the small amount of purified material usually available It is unlikely, however, that the larger and more irregular viruses, such as influenza, will ever be crystallized

The first crystals obtained were very small, but later, larger crystals of Type 1 (Mahoney strain) were obtained from a highly purified suspension in phosphate-saline solution which had been stored for a year at 4°C (Steere and Schaffer, 1958) These have recently been examined by X-ray crystallographic methods by Finch and Klug (1959) The crystals are rather unstable, and special techniques (the use of quartz capillaries and low temperatures) were necessary to handle them for X-ray work As discussed in Section III,C,2, these efforts were rewarded by the fact that the X-ray photographs obtained were of extremely high quality, indicating a remarkably greater degree of crystal perfection than has been hitherto reported for any other virus crystal.

The crystals are not cubic in symmetry but orthorhombic, i e , the unit cell has three axes at right angles, but these are not equivalent or even equal in length, the sides being 353, 378, and 320 Å Apart from the

* *Footnote added in proof.* This has now been achieved (Kaper, *J. Mol Biol*, in press).

particles at the corners of the cell there is one very close to the body center in an almost parallel orientation Moreover, since the sides of the unit cell are not very different from their mean value of 350 Å, the crystal lattice may be thought of as an approximately body-centered cubic one, similar to that of BSV, with an interparticle distance of 304 Å The relation of the crystal morphology to the lattice has been considered by Finch and Klug (1960)

In the case of the BSV and TYMV the cubic crystal symmetry implies that the particle symmetry must be at least tetrahedral and thus the particle is made up of a multiple of 12 subunits No such deduction is possible for poliovirus, the crystal is orthorhombic, which only implies that the virus has at least 2 subunits

However, the distributions of intensity on the diffraction photographs (Fig 17) show immediate evidence of a higher particle symmetry, and



FIG 17 Precession photograph of a poliomyelitis virus crystal taken with the X-ray beam approximately parallel to the side of the unit cell (Finch and Klug, 1959) [illegible]
within [illegible]
2-fold a[illegible]
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FIG 17 Precession photograph of a poliomyelitis virus crystal taken with the X-ray beam approximately parallel to the side of the unit cell (Finch and Klug, 1959) The beam has been set slightly off the axis to show more of reciprocal space within the limits of the precession angle (1½°) The orientations of the 5-, 3-, and 2-fold axes of the icosahedral point group 532 which lie in the basal reciprocal lattice plane are indicated by arrows The intensities of the crystal reflections tend to be strongest in these directions (cf Fig 15a)

here again it is icosahedral. Because the symmetry of the crystal lattice is a good deal lower than that of the individual virus particles, the actual point-group symmetry tends to be somewhat obscured by the "sampling" of the virus diffraction pattern on the reciprocal lattice of the crystal. But this difficulty is compensated for by there being more crystallographically independent views of the particle symmetry.

Icosahedral symmetry implies, of course, that the virus particle is built up out of 60 structurally equivalent asymmetric units. By measurements of the period of a slow modulation of intensity that is clearly discernible in the X-ray photographs along the "spike" directions, Finch and Klug concluded that the ratio of subunit size to particle diameter was similar to that for TYMV. They therefore suggested that the way in which the subunits are arranged to form the protein shell must be similar (Fig. 13).

Such physicochemical data as there are on poliovirus are given in the recent review by Schaffer and Schwerdt (1959). The particle weight has been reported as 6.7×10^6, but in the absence of diffusion measurements this figure must be regarded as only approximate. Of this about 25–30% is RNA (Schaffer and Schwerdt, 1959), so that the molecular weight of the structural unit is about 80,000.

It very likely, by analogy with the top component of TYMV, that the slower sedimenting, noninfectious, nucleic acid-free fraction C, which is found associated with the virus in infected tissue cultures, consists simply of empty protein shells. This would explain why electron micrographs of fraction C (Le Bouvier *et al.*, 1957) show particles of about the same size, but flatter and less sharply delineated than the intact virus particles. The still lighter noninfectious fractions observed, which are immunologically related to C, may consists of small groups of, or even isolated, subunits. The infectivity of the virus is, of course, associated with the RNA (Colter *et al.*, 1957).

II. Other Small Spherical Viruses

Preliminary X-ray studies have been made on three other spherical viruses. Some of the physical measurements on these viruses are presented in Table III.

1. Southern Bean Mosaic Virus (SBMV)

X-ray precession photographs of SBMV crystals have been obtained by Magdoff (1960). The crystals are orthorhombic with unit cell sides of $a = 295$ Å, $b = 508$ Å, $c = 474$ Å. Despite the large unit cell, the packing is apparently of a simple type. Since $b/a = \sqrt{3}$ and $c/a = 1.61$ ($\simeq \sqrt{8/3}$), the crystal lattice is approximately hexagonal close-packed with an interparticle distance of 295 Å, but there are presumably slight

differences in orientation or position which lead to the large orthorhombic cell

The photographs show "spikes" of intensity in the directions of the symmetry axes of the point group 532. These spikes appear to be much broader than those found in the case of the three viruses discussed above, showing that the actual arrangement of subunits may be rather different, but there is little doubt that the symmetry is icosahedral. An earlier statement (Franklin *et al*, 1959), based on very preliminary X-ray studies, that SBMV did not appear to have cubic symmetry, must therefore be revised

Magdoff has also obtained photographs of air-dried crystals and shown them to be monoclinic. The particles appear to be packed in sheets in pseudo-hexagonal array, in which the interparticle spacings are 250, 245, and 207 Å. While the first two distances agree nicely with electron microscope observations on SBMV crystals (Labaw and Wyckoff, 1957), the last one, if correct, implies that the particles are squashed or collapsed in one direction

2 Tobacco Ringspot Virus (TRV)

Crystals of this virus, prepared by Steere (1956) have been examined by Caspar and Langridge (unpublished). Only a limited number of X-ray photographs from dried crystals have been obtained. The diffracted intensity fades out beyond very low angles, but there is enough evidence to suggest that the crystal (and, therefore, the particle) has cubic symmetry. Wet crystals have not yet been examined

3 Coxsackie Virus

A preliminary X-ray investigation by Caspar and Langridge (unpublished) of orthorhombic crystals of this virus (Mattern and du Buy, 1956) has given an indication of some kind of symmetry, probably cubic. We should, of course, expect this because of the similarity of this virus to poliovirus. The unit cell is $a = 302$ Å, $b = 433$ Å, $c = 620$ Å, and the fact that these values are approximately in the ratio $1 : \sqrt{2} : 2$ suggests that the particles are packed to form a pseudo-face-centered cubic lattice. Nothing is known about the nature of the deviations from the strictly cubic lattice

I Comparisons with Electron Microscopy

1 Crystal Structure

There has been a certain amount of confusion in the past about the comparison of crystal structures deduced from X-ray analysis and by direct observation in the electron microscope. The results do not always

here again it is icosahedral. Because the symmetry of the crystal lattice is a good deal lower than that of the individual virus particles, the actual point-group symmetry tends to be somewhat obscured by the "sampling" of the virus diffraction pattern on the reciprocal lattice of the crystal But this difficulty is compensated for by there being more crystallographically independent views of the particle symmetry.

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H Other Small Spherical Viruses

Preliminary X-ray studies have been made on three other spherical viruses Some of the physical measurements on these viruses are presented in Table III

1 Southern Bean Mosaic Virus (SBMV)

X-ray precession photographs of SBMV crystals have been obtained by Magdoff (1960). The crystals are orthorhombic with unit cell sides of $a = 295$ Å, $b = 508$ Å, $c = 474$ Å Despite the large unit cell, the packing is apparently of a simple type Since $b/a = \sqrt{3}$ and $c/a = 1\ 61$ $(\simeq \sqrt{8/3})$, the crystal lattice is approximately hexagonal close-packed with an interparticle distance of 295 Å, but there are presumably slight

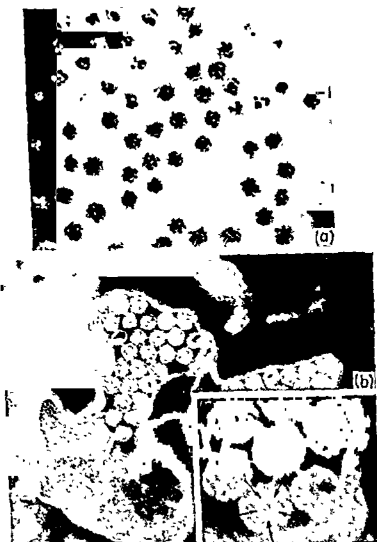


FIG 18 Electron micrographs of spherical viruses taken using the negative-staining technique of Brenner and Horne (1959)

(a) Turnip yellow mosaic virus (Brenner and Horne, 1959) Note the empty particles of *top* component Magnification × *230,000*

(b) Inclusion body from HeLa cells, 6 hours after infection with poliovirus Magnification × 176,000 (Horne and Nagington, 1960)

Inset Enlargement (Magnification × 600,000 showing instance of a ring of 5 knobs on the virus particle (Photographs supplied by R W Horne)

agree As we have seen above, X-ray analysis of wet crystals has revealed a variety of crystal structures, whereas electron micrographs (Labaw and Wyckoff, 1956a,b, Steere, 1957; Steere and Schaffer, 1958) invariably show a close-packed structure There is no paradox in this (Finch and Klug, 1959) Only a relatively small shear and shrinkage are required to convert a body-centered cubic structure, such as that of TYMV or poliovirus, to a close-packed one. It seems quite reasonable that, under the special treatment of the specimens necessary for electron microscopy (drying, freezing, examination under a high vacuum, etc), the particles should rearrange themselves to reach the close-packed structure The wet structure, which is held together by relatively weak forces, requires the presence of water and will naturally tend to collapse on drying

Where X-ray measurements have been made on dried crystals, there is much better agreement with electron microscope observations. This is the case for a derivative of tobacco necrosis virus (Cowan and Hodgkin, 1951), SBMV (B Magdoff, personal communication), and TRV (Caspar and Langridge, unpublished)

2 Particle Structure

We cannot review here all the results on the structure of small spherical viruses that have been obtained by the electron microscope, but it is relevant to discuss how far the results of X-ray analysis have been borne out by electron microscope observations Now that structures as small as 10 Å or less can be resolved with the electron microscope, it is becoming increasingly clear that the full power of the method depends, particularly in the case of biological structures, upon successful methods of specimen preparation, such as fixing, freeze-drying, and staining. Only recently has the electron microscopy of TMV, after a long history, come near to the visualization of the substructure deduced from the X-ray analysis (Section II,C)

We shall illustrate this point by reference to the case of TYMV By a frozen replica technique, Steere (1957) obtained electron micrographs showing a rather regular arrangement of knobs on the surface of the virus particle, but these knobs are spaced about 90 Å apart, and there are obviously less than 60 of them per particle. Likewise, in electron micrographs taken of TYMV (Brenner and Horne, 1959), using the phosphotungstic acid negative-staining technique, there is a marked granularity, but the number of knobs seems to be less than 60 (Fig 18a). This new technique has also clearly confirmed the hollow nature of the top component.

None of the micrographs mentioned gives any reliable information as to the symmetry of the virus, but Kaesberg (1956) has concluded, from

symmetry of the particle requires that these 60 units, whatever the detailed arrangement, must be in the form of 12 regular pentagons, and if such rings of 5 were consistently seen, the symmetry could be considered proved Only isolated instances of these have been observed by Horne and Nagington (Fig 18b, inset) The treatments involved in specimen preparation, however, may distort the symmetry of the particle without changing the number of subunits that can be seen on its surface If the number is found to be 60, or a multiple of it, it would be a most remarkable coincidence if the symmetry of the native virus were not icosahedral A virus with icosahedral symmetry need not, however, have 60 bumps or knobs discernible in electron micrographs (Fig 14)

J Icosahedral Features Observed by Electron Microscopy

Except for the systematic investigations of TYMV, the electron microscope and X-ray diffraction studies on other spherical viruses do not yet allow detailed comparison Electron micrographs of rabbit papilloma virus (Williams, 1953) provided the first definite indication of surface detail Williams (1953) observed that air-dried particles showed protuberances spaced about 70 Å apart A rough calculation from the diameter (450 Å) and inter-subunit distance, shows that the number is the order of 60, but this only suggests that the symmetry may be icosahedral However, electron microscopy has provided direct evidence of icosahedral features in the case of four, widely different viruses, namely, the small bacteriophage ϕX-174 (Hall *et al*, 1959), an insect virus, Tipula iridescent virus (Williams and Smith, 1958), an adenovirus (Horne *et al*, 1959a), and polyoma virus (Wildy *et al*, 1960)

1 ϕX-174

This small bacteriophage of (dry) diameter about 250 Å is of great interest because the DNA it contains has been recently shown to be single-stranded (Sinsheimer, 1959a,b) Electron micrographs (Hall *et al*, 1959) show that the particle has a knobbly surface One knob lies slightly above an approximately regular ring of five neighbors surrounding it [Fig 19(2)] This is exactly what would be observed if there were 12 knobs arranged at the vertexes of an icosahedron Notice that if these knobs represented the asymmetric units, the structure would not have strictly icosahedral symmetry Only if each knob consisted of 5 units (see p 302) could this be the case The particle would then have the expected 60 structural subunits

a study of the shadows cast by TYMV particles, that the virus particle has the approximate shape of a regular icosahedron Thus, in respect of symmetry, Kaesberg's observations agree with the model put forward on the basis of the X-ray results; but analysis of the shadows from such small particles must be regarded with caution [see the remarks in Williams and Smith (1958)]

Very recently Huxley and Zubay (1960) and Nixon and Gibbs (1960) have examined TYMV in the electron microscope by the negative staining method and have observed regularly disposed protuberances on the surface of the particles These are the morphological subunits forming the protein shell. From an analysis of the characteristic patterns of knobs observed in different views of the particles and using the fact, known from the X-ray results (Klug *et al*, 1957b), that the particles must have at least tetrahedral symmetry, Huxley and Zubay (1960) showed that there are 32 such morphological subunits. By a careful study of the electron micrographs, they concluded that the arrangement which accounted for the patterns seen was best approximated to by a polyhedral model in which 12 of the units lie at the vertexes of an icosahedron and 20 at the vertexes of a pentagonal dodecahedron Nixon and Gibbs (1960) came to the same conclusion Since approximations to polyhedra of this type cannot be distinguished in the electron micrographs, it is the X-ray evidence that leads to the conclusion that the 32 vertex polyhedron has true 532 symmetry. This means that the 32 morphological units seen in the electron micrographs must be further subdivided in accordance with the principles discussed in Section III,D,3

The fact that there are 32 morphological units indicates that the 60 crystallographic units may be subdivided into two or three chemical units (Klug and Finch, 1960) This conclusion is consistent with the preliminary end group analyses (J I Harris, personal communication) It now seems likely that the combination of X-ray, electron microscope, and biochemical studies can provide the information necessary to deduce the actual arrangement of the protein subunits in this spherical virus

Another instance where agreement may be reached with the X-ray results is in the case of poliovirus. Using the negative-staining technique, Horne and Nagington (1959) have taken electron micrographs of this virus (Fig 18b) which show a fairly large number of knobs, of the order of 20 to 30 Since this represents only half the view of the particle, it seems that these observations may actually resolve the 60 asymmetric units indicated by the X-ray results The icosahedral

symmetry of the particle requires that these 60 units, whatever the detailed arrangement, must be in the form of 12 regular pentagons, and if such rings of 5 were consistently seen, the symmetry could be considered proved Only isolated instances of these have been observed by Horne and Nagington (Fig 18b, inset) The treatments involved in specimen preparation, however, may distort the symmetry of the particle without changing the number of subunits that can be seen on its surface If the number is found to be 60, or a multiple of it, it would be a most remarkable coincidence if the symmetry of the native virus were not icosahedral A virus with icosahedral symmetry need not, however, have 60 bumps or knobs discernible in electron micrographs (Fig 14)

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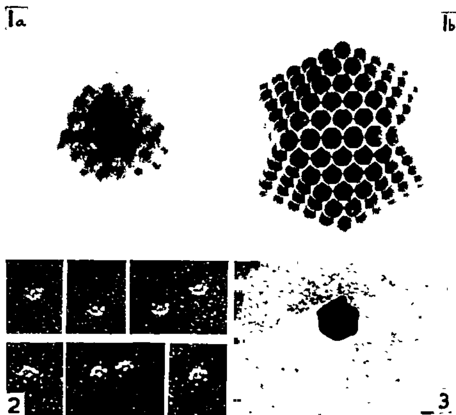


FIG 19 Electron micrographs of viruses exhibiting icosahedral features

(1a) Adenovirus particle (Type 5) at high magnification (× 600,000 Negatively stained with phosphotungstic acid (1b) Model of icosahedron in the same orientation (Horne *et al*, 1959)

(2) Bacteriophage ϕX-174 shadowed at angle 1 1 Magnification × 160,000 (Hall *et al*, 1959)

(3) Tipula iridescent virus A frozen-dried, doubly shadowed particle Magnification × 85,000 (Williams and Smith, 1958) These shadows could only have been cast by a particle with the shape of an icosahedron (Photographs supplied by R W Horne, C E Hall, and K M Smith, respectively)

2 *Tipula Iridescent Virus*

This is a very large virus with a frozen-dried diameter of about 1300 Å. Williams and Smith (1958) were able to deduce unambiguously, from the shapes of the shadows cast in electron micrographs of particles

unresolved subunits. It is difficult to understand how the faces could appear so sharp unless they were made up of small subunits packed in a regular way. A regular arrangement of subunits has now been seen in particles of virus grown from a different host (Smith and Hills, 1959)

3 *Adenovirus (Type 6)*

This virus, of diameter about 800 Å, has been shown by electron microscope observations (Horne *et al*, 1959) to be icosahedral in form, the subunits on the surface of the virus having been clearly resolved by the negative-staining technique. An electron micrograph is reproduced in Fig. 19(1a) and a schematic model in Fig 19(1b) The shape of the virus is clearly like that of an icosahedron, with 6 subunits along an edge so that the total number is 252. The symmetry of an arrangement of asymmetric units like that of Fig 19(1b) is not necessarily icosahedral, because all the subunits are not equivalent There is more than one kind of environment for a subunit. it may be situated in a face, on an edge, or at a vertex, and in each case the arrangements of neighbors is different This point is also demonstrated by the fact that the number of subunits is not a multiple of 60, as would be required for icosahedral symmetry.

4 *Polyoma Virus*

A recent detailed electron microscope study of polyoma virus by Wildy *et al.* (1960) has established that this virus consists of 42 morphological subunits. Their arrangement appears to be like that shown in Fig 14a A particularly interesting aspect of this study is that substructure has been detected in the morphological units Wildy *et al.* (1960) conclude that there are 12 pentagonal units on 5-fold axes and 30 hexagonal units on 2-fold axes. The symmetry of the particle thus clearly appears to be icosahedral even though it does not have the shape of an icosahedron Moreover, the total number of subunits suggested by the electron micrographs is $5 \times 12 + 6 \times 30 = 240$ which is 4×60

K. *Icosahedral Symmetry and Virus Structure*

Viruses having widely different host range—animal, plant, insect, and bacterium—have structures which exhibit, in one way or another, features which may be called icosahedral These viruses fall into two classes.

(a) Small viruses, such as the spherical plant viruses or poliovirus, which have a diameter about 300 Å, and which appear to be built out of 60, or a multiple of 60, subunits arranged according to icosahedral symmetry. This does not imply that the particles need look much like an

icosahedron (Fig. 13). The bacteriophage ϕX-174 may also belong to this class.

(b) Larger viruses, such as adenovirus and TIV, which actually have the shape of an icosahedron, the faces of which seem to be built up of a regular array of small subunits.

1. Small Spherical Viruses

The prediction of Crick and Watson (1956) that the small viruses should have cubic symmetry (Sections I,C and III,D) has been amply borne out. What could not, however, have been readily foreseen is that only icosahedral symmetry would be found in the spherical viruses whose structure has been analyzed. The question has been raised (Finch and Klug, 1959) whether there is not another general principle at work here, and one to be added to those already put forward by Crick and Watson.

The reasons why one might expect small viruses to be made up of subunits have been clearly stated by Crick and Watson (1957) and recapitulated in Section I,C above. The nucleic acid controls the formation of virus protein, and on current views of protein synthesis there will be a limit to the length of polypeptide chain produced set by the nucleic acid of the virus. Another argument was given by Crane (1950), who pointed out that small subunits would be favored in an evolutionary process because there would be less wastage due to mistakes in synthesis.

Whatever the reason, if a protein framework of a certain size is to be made up of a set of small identical molecules, arranged to form a shell with cubic symmetry, the size of the individual subunits will be smallest if icosahedral symmetry is used. This is because it allows the use of the greatest possible number, namely, 60, of identical asymmetric units to build a framework in which they are also identically packed.

Since each asymmetric unit may consist of a number of chemical subunits, the total number of chemical subunits may be a multiple of 60. These smaller units, even if identical, would not be structurally equivalent since their environments would not all be the same. It could not be recognized in a simple manner by X-ray diffraction that these identical but differently arranged molecules really have the same structure. This would only show up when a complete structure analysis has been made.

The arguments for the icosahedral symmetry in small spherical particles are a good deal weaker than those for cubic symmetry in general. Thus a small particle, half the diameter of the virus particles we have

may be the case of ferritin (Harrison, 1959), a particle of diameter about 180 Å. Ferritin is in some ways analogous to a virus since it consists of a protein coat containing a central core of iron Harrison concludes that the symmetry is probably octahedral

2. *Larger Icosahedral-Shaped Viruses*

The argument presented above for the occurrence of icosahedral symmetry in virus structures is essentially from a principle of economy, i e, it is favorable for the use of small subunits But the size of a particle which may be built out of 60 structural units of molecular weight about 20,000–60,000 is rather limited The diameter of any reasonably compact arrangement could not be much greater than 300 Å

To build a particle of twice the diameter out of units of the same size, four times as many would be required Since 60 is the maximum number of structurally equivalent units that can be placed on the surface of a sphere, four different kinds of environment would ensue, and four different sets of contacts would have to be used The question is, can some more efficient way of packing this large number of units be found? It is likely that the answer to this structural problem is to be found in the type of arrangement clearly established in the case of adenovirus and indicated in that of TIV

IV. Conclusions

It may be worthwhile emphasizing some points that are implicit in this review. The investigations of substructure in both TMV and the crystalline spherical viruses, but particularly in the case of the former, have already made possible some generalizations about the way in which small viruses are put together It seems likely that the parts are made by a type of subassembly process before being assembled to build the virus. The forces holding together the protein subunits in the virus particle are like those between globular protein molecules in a crystal The configuration of the RNA is determined by its regular packing with the protein, and we may think of the process by which the protein subunits and the RNA come together to form the regular structure as a kind of "co-crystallization" of two very unlike components The mechanism of infection presumably involves the reverse process After some time in the environment of a susceptible cell the particle breaks down to expose the infectious nucleic acid

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BASIC MECHANISMS OF NONSPECIFIC RESISTANCE TO VIRUSES IN ANIMALS AND MAN

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I. Introduction

Natural resistance or natural immunity denotes a congenital state of nonsusceptibility to a given infection, not dependent on earlier spontaneous or experimental contact with the responsible agent or its antigens Natural immunity is not conferred by immunological mechanisms, but represents a species-specific property of a given animal, and it is related in a more predictable way than acquired immunity to hereditary, sex, hormonal and growth factors It is well known that man is completely nonsusceptible to a host of virus infections of domestic animals, which in turn are not susceptible to many human virus infections

The essential aspects of the mechanisms of antiviral and antibacterial immunity are intimately linked with the fundamental properties of the pathogenesis of viral and bacterial infection

The basis of innate (species) immunity to bacterial infection lies in

the low sensitivity of the nonsusceptible organisms to the toxic metabolites of a given microorganism, and in the active depression of its multiplication through the mechanisms of phagocytosis and nonspecific lysis, exemplified by the presence of complement, properdin, lysozyme, and β-lysine. It is known that in the main the pathogenic microorganisms multiply in the highly nutrient tissue fluids which bathe the organs and tissues of man and animals. Equipped with an arsenal of aggression (in the form of toxicity, toxigenicity, encapsulation, hyaluronidase), they become less susceptible to the destructive effects of phagocytosis, of specific and nonspecific lysis, and of other protective devices of the host than nonpathogenic microorganisms. A variety of external and internal processes inimical to the microorganism serves to weaken or paralyze these protective mechanisms, thereby fundamentally affecting the severity of the infective process, and can even facilitate the multiplication of nonpathogenic bacteria; this is in fact regularly observed during the agonal period.

It is well known that the singular nature of the protective factors of immunity is specifically linked to the nature of the microbial stimulus. The individual and species-specific variations of the microorganism reacting to it are of little consequence, the immunological changes always strictly corresponding quantitatively to the microbial stimulus

Thus the antitoxic immunity is not provoked by the microbial agents, not producing the exotoxin as also the development of specific phagocytic immunity is not possible without the participation of specific antigens, which form an innate part of the bacterial cell

It is understandable that virus infections, associated with a particular group of agents, which are highly specific in respect to their biological and morphological characteristics, should have their own peculiarities of pathogenesis and immunity, that differ from those of bacterial infections in a number of important respects.

The causal organisms of viral infections differ radically from the pathogenic bacteria in their obligatory intracellular parasitism, which is adopted in its peculiar way to selected tissues or organs of the body. For this reason the multiplication of the viral agents in the human or animal organism can only proceed in tissues which provide especially favorable conditions for the intracellular parasitism of the organism. In the absence of such susceptible tissues there is a condition of total nonsusceptibility to the particular organism of a characteristic strength: such a species-specific congenital immunity can neither be diminished nor augmented by varying the dose of the evocator, nor does it diminish when the general resistance of the animal is lowered

Thus the susceptibility of man or animals toward a given virus implies

first the existence of a cell complex within which reproduction of the causative organism can proceed, and second a sensitivity on the part of these cells and of others in the system of the organism to the toxic substances of the virus and to the toxic action of the virus proteins. The latter leads to a disorganization of neurohumoral control and of the normal metabolism of the susceptible tissue The degree of susceptibility of animals to different viruses varies greatly. *It often happens* that the virus is continually multiplying for a prolonged period in specific animal tissues without completely destroying the normal physiological functions of the organism. This is associated with relatively less active multiplication of the virus in the susceptible cells, together with relatively slight damage to the latter by the toxic substances of the virus, as well as by the products of the altered cell metabolism (Smorodintsev, 1948, 1951, 1955)

Thus, in the case of the tick *Ixodes persulcatus* one observes intensive reproduction and trans-ovarial transmission of tick-borne encephalitis which can continue for many years in successive generations, at all stages of development of the tick, without any physiological effect on it, nor in particular on its proliferation Analogous examples of protected symbiosis with the animal or human organism can be observed, in the case of herpes virus in man, with Taylor's virus in mice, etc

Many viruses affecting man provoke a form of symptomless infectious process in laboratory animals or in tissue cultures, which proceeds without clinical or histological changes The observation of the proliferation of such viruses in the organism of infected animals or in tissue cultures is made possible with the help of other biological models more sensitive to the given virus, or by means of serological methods, interference phenomenon, and fluorescent antibody techniques

More frequently cases arise where the infected tissues react to the presence of virus particles or their toxic components by way of necrosis, with the simultaneous manifestation of clinical indications of acute or chronic morbid processes

The reproduction of viruses is observed at its best in cells and tissues at the height of their physiological function and metabolism For this reason the administration of metabolic stimulators (cortisone) does not ameliorate the activity of virus infection, but on the contrary only exacerbates it Lowering of the general resistance (reactivity) of laboratory animals does not intensify, but, on the contrary, diminishes, the rate of reproduction of the virus in the organism, as, for example, through starvation, intoxication, burns, and bacterial infection X-rays are an exception, producing radiation sickness, which provokes equally both bacterial and virus infection

The characteristic peculiarity of natural immunity to virus infectic thus lies in the absence of a benign environment for the multiplicatio of the virus in the organism, in other words, the absence of tissue cel suitable for the intracellular parasitism of a given virus. Such a com plete nonsensitivity is linked through a series of translations with a stat of high susceptibility. In cases of partial sensitivity the reproductior of the virus can proceed within the sensitive cells at slow and at rapic rates, without significant disorganization of the normal cell metabolism or any concomitant morphological effect.

The mechanism of the different pathogenicity of the virus is not fully understood, and it can be studied in considerable detail by tissue culture methods, using cultures infected with the virus, which multiplies with various intensities in the susceptible cells, with or without the manifestation of cytopathogenic effects

For the full understanding of the mechanism of natural resistance of animals to virus infections, it is necessary to uncover the nature of the protective factors which limit partially or completely the possibility of reproduction of the virus, and of the related development of the pathogenic reaction of the host organism.

If there is a complete absence of cells susceptible to a given virus in the animal organism, capable of sustaining its multiplication, the development of virus infection cannot proceed, and the causative organism is bound to die out However, the period of extinction will differ considerably with different viruses, and can vary from a few hours to several weeks.

The question naturally arises as to what is responsible for the different rates of extinction of different viruses in the tissues of nonsusceptible animals, and what is the nature of the protective factors causing complete extinction of the virus in the susceptible organism, or differences in the severity of infection in susceptible organisms

During the study of this problem, extending over several decades, there has been a tendency to postulate a similarity or even identity of protective factors in antibacterial immunity and in antiviral immunity Supporters of this theory have assumed that the mechanisms leading to the destruction of viruses in the naturally resistant organism of warm-blooded animals and man were based on the same cellular and humoral factors which were discovered by Mechnikov, Ehrlich, Bordet, and other scientists when studying antibacterial immunity. The rapid elimination of viruses in naturally resistant organisms was explained by the action of phagocytosis or nonspecific lysis elicited by complement, lysozyme, and β-lysine, with special emphasis given, during the last few years, to

the presence of Pillemer's properdin, participating also in the destruction of viruses.

The systematic study of this problem in our laboratories since 1938 has revealed significant differences between the mechanisms of natural immunity against viruses and the protective factors of antibacterial immunity, which is indeed to be expected from the highly characteristic nature of the biology of viruses, and of the pathogenesis of virus infection

Nonspecific cell factors as well as humoral and general physiological factors enter into the mechanism of natural (species-specific) antiviral immunity, whose properties are far removed from the mechanism of antibacterial immunity These same nonspecific protective factors play their role in contributing a specific component in the development of acquired immunity following an acute viral infection, which frequently confers a pronounced immunity of long duration, but they also play an important part in chronic virus infections characterized by a low degree of specific immunity, or even by complete absence of it

II Natural Susceptibility and Nonsusceptibility, and Their Relation to Hereditary, Developmental, and Hormonal Factors

The susceptibility or resistance of the cellular system of the organism to a given virus, its ability to sustain the multiplication and development of the infective process, depends in the first instance on congenital factors In cases where the resistance of the species to a particular virus under study is relative rather than absolute, it is possible to vary it by a process of selection of the more sensitive, or conversely, of the more resistant strains Successive generations of such strains selected by such principles leads to the development of "sensitive" or "resistant" variants of animals, and such properties are not universally developed toward all viruses, but only toward the virus or group of related viruses which are the subject of the particular investigation In his classic work Webster (1937) obtained artificial strains of mice resistant to intestinal bacteria; these strains showed no resistance toward neurotropic viruses, and conversely strains selected for their resistance to neurotropic viruses showed specific resistance only toward that particular group of infectives

Comparing two strains of white mice (Swiss and PRI) which show different resistance toward a vaccine strain of yellow fever 17D, Sabin (1952) observed a different rate of multiplication of the virus in the brain of the two strains, being maximal in the Swiss strain, and minimal in PRI, which was explained by the authors by postulating two complementary genes in the resistant strains, associated with depressed

proliferation On the other hand, it was observed that newborn mice of type PRI showed pronounced sensitivity to virus 17D, despite the fact that quantitative measurements did not show increased multiplication of virus in the brain of the adult resistant mice. The difference was shown to lie in the fact that in the newborn mice the virus destroyed the brain cells, while these are not susceptible to the same type of virus in adult mice. Thus a heightened sensitivity of the cells toward the cytopathogenic action of the virus can depress the protective effect of inherited resistance. The inherited resistance of mice toward a given group of viruses can be overcome by a process of adaptation of such pathogens to the resistant strain, giving rise to a virus variant with greater cell pathogenicity, or possessing more intensive powers of multiplication, by virtue of which the virus compensates for the inherited resistance Such are the neurotropic strains of the viruses of influenza, poliomyelitis, and dengue, which multiply in the brain of white mice after preliminary adaptation to the different mode of life, and such are the new antigenic variants of influenza virus arising during multiplication of the virus in animals and man with homologous specific immunity.

Resistance toward virus infection often depends on developmental factors, generally intensifying it in the adult animal and in man, and minimizing it in the newborn In adult mice extreme susceptibility of the central nervous system (CNS) to viruses can be coupled with high resistance to peripheral extraneural infection with the viruses of St. Louis or Japanese encephalitis, according to Sabin (1952) this resistance is linked with the development of anatomical barriers, that interfere with the dissemination of the virus along neural pathways Cases are known in which developmental resistance toward neurotropic viruses is evidenced by a high resistance in adult animals toward the multiplication of the virus. White rats and guinea pigs during the first few days after birth are highly susceptible to the virus of tick-borne encephalitis, while in the adult animal multiplication of the virus is either completely suppressed, or occurs at such low levels that it excludes the development of morphological or clinical changes The mechanism of developmental resistance is not clear, and is possibly linked to the appearance in adult tissues of supplementary enzymes, which interfere with the synthesis of virus particles. Thus, in the brain of the newborn rat there is a deficiency of three enzymes—succinic dehydrogenase, cytochrome oxidase, and adenosinetriphosphatase (Potter *et al*, 1950)—which possibly facilitates the multiplication of the virus, thereby enhancing susceptibility, while the emergence of these enzymes may facilitate the development of resistance. An interesting fact was observed by Rowe (1953) with Coxsackie virus, which is known to develop well in the skeletal muscular

tissue of newborn mice during the first days after birth, but not in the musculature of adult mice. After denervation of the adult muscle, Coxsackie virus multiplies well in infected fibers. It is possible that in this case also the change in susceptibility may be bound up with the change in enzymatic pattern, consequent upon the cessation of production of phosphorylase and phosphoglucomutase, which are present in low concentration in the muscle of embryonic and neonatal mice.

The natural resistance to viral agencies can be altered substantially under the influence of hormonal factors. The susceptibility of female monkeys to poliomyelitis is greatly increased after castration, while the administration of estrogens to the castrated animals prevents the increased susceptibility (Aycock, 1936) Analogous changes are observed with hamsters. castration results in enhanced susceptibility toward intracranial or intraperitoneal infection with poliomyelitis, which is suppressed by the administration of testosterone or gonadotropic hormone. Administration of estrogen increases the resistance of the animals to vaccine virus (Sprunt and McDearman, 1940). Thyroxine lowers the resistance of mice to the virus of mouse pneumonia (Weiss *et al*, 1952).

Of special significance is the effect on natural resistance obtained with adrenocorticotropic hormone (ACTH) and cortisone ACTH sharply increases the susceptibility of monkeys to poliomyelitis and of mice to influenza virus. Cortisone enhances the susceptibility of a number of animals to neurotropic viruses, e g., of monkeys and hamsters to poliomyelitis (Schwartzman and Fisher, 1952; Sabin, 1952). Adult mice, which are fully resistant to infection with Coxsackie virus, become as susceptible under the influence of cortisone as newborn mice Cortisone also increases the susceptibility of chick embryo to the viruses of influenza and parotitis, producing a 2- to 6-fold yield increase compared with untreated eggs. (Kilbourne and Horsfall, 1951). The stimulating effect of cortisone on the reproduction of pathogenic viruses is evidently connected with the changes in protein metabolism, which stimulate the synthesis of virus particles A similar stimulatory effect on the multiplication of viruses in partly resistant and in susceptible animals follows their irradiation with X-rays (De Gara and Furth, 1945; Syverton *et al.*, 1952; Cheever, 1953; Sivertseva, 1955, Al A Smorodintsev, 1956, 1957; Cajal *et al*, 1959; Skhanskaya, 1957). Even prior to the development of radiation sickness, the multiplication of viruses is significantly increased in the susceptible tissues, and the local protective reactions of the infected cells are diminished (Al A. Smorodintsev, 1957). This stimulating effect of X-rays is a primary one, and only later does a general depression of immunological reactivity supervene. As in the case of cortisone, the mechanism is inadequately understood, and it is evidently

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of the virus-neutralizing inhibitors than is possessed by complement (Smorodintsev and Luzyanina, 1958, Polyak *et al*, 1959).

The quantitative measures of virus-neutralizing activity of the thermolabile inhibitors, obtained in parallel experiments with natural and heated serum are fully in agreement with the activity of immune sera, showing their characteristic high protective effects.

The influenza virus strains sensitive to the action of thermolabile inhibitors are neutralized by the normal serum of guinea pigs, white mice, and rabbits in the hemagglutination test with 2 HE virus in dilutions of 1.80—1:320.

In experiments on susceptible white mice or chick embryos, 1 ml of serum neutralizes up to 10 million infective doses of virus.

The activity of thermolabile inhibitors is not related directly or indirectly to complement, since the virus-neutralizing activity of the normal sera investigated is not changed by the preliminary removal of complement from the system antigen-plus-antibody or by a suspension of formalinized erythrocytes sensitized with hemolysin (Smorodintsev and Shishkina, 1951).

The intensity of the virus-neutralizing activity of normal thermolabile inhibitors toward influenza virus depends on the biological properties of the virus under study, and frequently also on its virulence in white mice (Table I) The normal serum of white mice actively

TABLE I

ACTION OF THE SERUM OF WHITE MICE ON THE VIRULENT AND NONVIRULENT VARIANTS OF STRAIN-3711 OF A' INFLUENZA IN CHICK EMBRYOS

Serum used	Infectivity titer of virus (log 10)	
	Virulent	Nonvirulent
None (control)	7.5	7.5
Heated, undiluted	8.0	6.5
Heated, diluted 1 3	8.0	6.5
Unheated, undiluted	8.0	1.5
Unheated, diluted 1 3	8.0	4.0

neutralizes nonpathogenic strains of influenza virus possessing no adaptation for mice, but it has little or no effect on adapted (pathogenic) strains (Chu, 1951, Briody *et al*, 1955; Smorodintsev and Luzyanina, 1958).

With progressive adaptation of nonvirulent strains of influenza virus toward white mice and increasing virulence, there is a progressive in-

linked with changes of normal metabolism which are beneficial to the synthesis of viruses.

Under the influence of X-ray irradiation the susceptibility of animals to many viruses increases significantly. This demonstrates the greater intensity of reproduction of viruses in the susceptible tissue, and the increased severity of such infections (shortened period of life and increased mortality), which are in proportion to the dosage of irradiation The period of maximum lowering of resistance to virus infection occurs between the third and twelfth days of radiation sickness. In the irradiated organisms virus infections running an acute course adopt a prolonged subchronic pattern, with a rather more prolonged period during which the virus continues to multiply in the surviving animals. A major role in the mechanism of increasing the severity is played not only by the lowering of immunogenesis, but also by a change in the nonspecific defense factors, which facilitates the more intensive multiplication of the virus.

The depression of reactive defense processes in the cells of irradiated animals, directed at the limitation of spread and elimination of the virus, is frequently characterized by a marked reduction in the formation of oxyphilic substances around the microcolonies of virus. The metabolism of the susceptible tissue is altered in a direction more favorable to the synthesis of virus particles

Unlike the properdin, which disappears rapidly from the bloodstream of irradiated animals, the level of virus-neutralizing, thermolabile inhibitors remains unchanged Only when the intensity of irradiation reaches 800 to 1600 r is there a reduction in the thermolabile substances in the blood of animals during the agonal period of radiation sickness.

III. Virus-neutralizing Inhibitors of Native Serum, Their Biochemical Nature and Their Relation to Properdin

Thermolabile, nonspecific inhibitors are found in the unheated serum of many warm-blooded animals, possessing marked virus-neutralizing activity (Ginsberg and Horsfall, 1949; Smorodintsev and Shishkina, 1950, 1951). The normal serum of white rats, mice, guinea pigs, rabbits, sheep, cattle, hens, and other animals has an intense power of neutralizing the hemagglutinative and infective activity of the influenza virus and of a series of other viruses, when acting under the experimental conditions employed for titrating the virus-neutralizing activity of immune area. This activity of serum is only partly destroyed by heating for 30 minutes at 56°C. and requires heating at 62°C for 60 minutes for complete destruction, which shows a higher degree of thermal resistance

the sera of various laboratory animals (rabbits, guinea pigs, white mice), and their relationship to the properdin of Pillemer

During fractionation of the proteins of normal sera of rabbits, guinea pigs, and white mice according to the method of Cohn, the virus-neutralizing activity appears to be concentrated in fraction III-0, accounting for the initial activity of the natural sera in the biological neutralization and hemagglutinin-inhibition reaction.

The further analysis of fraction III-0 resulted in the separation from it of a β-lipoprotein fraction, a protein component containing the initial serum activity, while the lipoid component containing 50% of the total serum cholesterol, which was obtained by repeated extraction with alcohol and ether, was devoid of activity (Polyak, 1960) Electrophoretic study of the β-lipoprotein fraction shows it to be homogeneous and close in mobility to γ-globulin

It was found possible to cleave the β-lipoprotein into two protein components by freezing the fraction at $-10°C$ and then thawing out Approximately 90% of the protein from this fraction and all the cholesterol remain in the supernatant, while the sediment separated by centrifuging for 60 minutes at 4000 r p m contains some 10% of the protein, in which are concentrated the nonspecific thermolabile inhibitors. As the β-lipoprotein fraction contains some 10% of the total serum protein, the active protein component of this fraction, obtained as a deposit after successive freezing and thawing, represents only some 1% of the original serum protein Unlike the case of the rabbit, the virus-neutralizing inhibitor of the serum of guinea pigs and white mice could not be obtained as a deposit after treatment analogous to that applied to the β-lipoprotein fraction, which is a consequence of species differences in the serum protein of different animals

The virus-neutralizing inhibitors of the serum of white rats are characterized by a number of biochemical peculiarities, and they are present in normal sera in negligible amounts When fractionating according to Cohn, the inhibitors of the white rat are not found in the III-0 fraction, but in the fraction I + III-3, and it is necessary to have prolonged prior contact with the sensitive strain of influenza virus under study (1 hour at 37°C or 18 hours at 0°-4°C), otherwise the results of biological neutralization or hemagglutinin-inhibition reaction are slight or even absent

Trypsin, which has no action on the virus-neutralizing activity of normal serum (Ginsberg and Horsfall, 1949, Chu, 1951), destroys the activity of the β-lipoprotein fraction

Lecithinlike inhibitors of influenza virus described by Utz (1948, 1949)

crease in the resistance of the virus toward the action of thermolabile inhibitors of serum (Smorodintsev, 1957b; Smorodintsev and Luzyanina, 1958)

Thermolabile inhibitors retain their activity at a temperature of —10°C and lower for periods of many months. According to the data of Ginsberg and Horsfall (1949) the presence of Ca^{++} ions is required before any activity is shown The complex with virus appears reversible by addition of sodium citrate, or washing off from the serum on a fine filter surface, or treatment with fluorocarbon.

Viruses which are susceptible to the action of thermolabile inhibitors combine firmly with them, and in doing so they remove the virus-neutralizing activity of natural sera under the same conditions of contact and quantitative correlation of virus to inhibitor, which are observed in removal of antibody from immune sera by adsorption with specific virus.

Cross-immunity experiments showed the uniformity of the nature of the thermolabile inhibitors for such widely separated groups of viruses as influenza virus and phage, which would seem to point to a general action of the active substrate on different viruses

The full range of action of thermolabile inhibitors on various groups of viruses has not been determined, but even the existing data indicate it to be very wide, embracing the whole group of myxoviruses, adenoviruses, the ECHO viruses, Coxsackie, transmissible encephalitis, poliomyelitis, and bacteriophages In each group there are to be found both sensitive and more or less completely resistant strains (Smorodintsev *et al.*, in press) The resistance of the latter against the action of natural serum does not extend to other strains of the same group. There is a pronounced correlation between the measure of sensitivity of various viruses to thermolabile inhibitors, and their behavior in the organism of susceptible and nonsusceptible animals.

The introduction into resistant animals of viruses highly susceptible to thermolabile inhibitors is followed by their disappearance within a few hours from the site of injection, from the blood, and from the internal organs

In susceptible animals, viruses which are sensitive to the inhibitors produce a mild infection of a benign or symptomless nature, which points both to the low virulence of the virus and also the high nonspecific resistance of the organism.

Investigations in our laboratories (Luzyanina and Polyak, 1958; Polyak, *et al.*, 1959; Polyak, 1960) have defined more closely the biochemical nature of nonspecific, virus-neutralizing inhibitors contained in

the sera of various laboratory animals (rabbits, guinea pigs, white mice), and their relationship to the properdin of Pillemer

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are quite different from the β-lipoproteins in their biochemical properties, and also in that the neutralization effect operates only at 37°C, while the β-lipoprotein is active in interacting with influenza virus in the cold (+4°C).

The high biological activity of β-lipoprotein is its chief characteristic, sharply differentiating it from many thermostable inhibitors, most of which are practically inert under the *in vivo* conditions of the experiment. The virus-neutralizing thermolabile inhibitors, in concentrations of 5-10 mg /ml., are easily adsorbed by zymosan from the sera of rabbits, guinea pigs, and white mice They retain their biological activity for long periods both in the intact serum and in the β-lipoprotein fraction at +4°C. and especially at −10°C. and lower.

The activity of the virus-neutralizing thermolabile inhibitors is retained in the presence of lowered ionic concentrations produced after prolonged dialysis over a period of 3–5 hours against distilled water, and also in the absence of complement The thermal resistance of the virus-neutralizing inhibitors increases as the salt concentration is reduced.

After treatment of the serum according to Cohn, the various components of complement are found outside the β-lipoprotein fraction, and its biological activity does not suffer thereby.

The highest virus-neutralizing activity is possessed by the serum of rabbits, while that of guinea pigs and white mice is somewhat less, with the serum of white rats showing the least activity.

Our investigations have revealed the essential differences between the nonspecific, thermolabile, virus-neutralizing inhibitors and the properdin of Pillemer, to which many authors ascribe not only bactericidal activity, but also virus-neutralizing activity (Ginsberg and Wedgwood, 1960). The singular and independent nature of the thermolabile virus-neutralizing inhibitors, and the differences between these inhibitors and properdin, can be deduced from the following factors:

(*1*) According to the data of Pillemer *et al* (1954) and Pillemer (1955), the largest amount of properdin is contained in the serum of white rats (50 units), and the least amount in the serum of rabbits (2–4 units) and especially of guinea pigs (up to 2 units). In conformity with this, the serum of guinea pigs is used as a source of complement in the quantitative titration of properdin, neglecting the slight quantities of properdin in the serum

The same animals differ considerably in respect to virus-neutralizing inhibitor content in their serum. The smallest amounts of inhibitor are contained in the serum of white rats, which are particularly rich in properdin. One milligram of normal serum of white rats neutralizes not more than 100–1000 infectious doses of virus for chick embryo,

while 1 ml. of guinea pig or rabbit serum, containing mere traces of properdin, neutralizes 1–10 million infective doses of susceptible influenza virus strains

(2) The treatment of natural sera with cultures of Flexner dysentery bacilli completely removes their properdin, which can then no longer be determined in bactericidal tests with susceptible dysentery cultures However, the removal of the properdin has no effect on the content of virus-neutralizing inhibitors in relation to strains of influenza or other viruses sensitive to them (T J. Luzyanina, in press).

If the natural sera are treated with a suspension of influenza virus, all the virus-neutralizing inhibitors are removed, but the properdin content and the bactericidal activity associated with it remain unaffected These experiments were carried out by us with sera from white mice and rabbits, and also with a mixture of equal parts of normal sera of white rats (source of properdin) and guinea pigs (source of virus-neutralizing inhibitor).

(3) The bactericidal activity of properdin is dependent on the presence of all four components of complement, which, together with magnesium ions, enter into the constitution of the properdin system.

On the other hand, the β-lipoprotein fractions, as also the original normal serum, retain their initial virus-neutralizing activity after preliminary removal of all the complement Under the conditions of fractionation of the normal serum according to Cohn, the β-lipoprotein fractions does not contain the complement fractions C_1 and C_4.

(4) The biochemical composition of properdin differs radically from that of the thermolabile virus-neutralizing inhibitors According to the data of Pillemer (1955, Pillemer *et al*, 1954) properdin appears to be a euglobulin (β-globulin). According to more recent data, properdin does not appear to be a homogeneous fraction, but represents a γ-globulin, containing 30–40% α- and β-globulins (Ishker and Linder, 1956; Tovarnitsky, 1959).

As already shown above, upon fractionation, according to Cohn, and by electrophoresis the thermolabile, virus-neutralizing inhibitors of normal serum of rabbits, guinea pigs, and white mice behave as β-lipoproteins.

(5) The activity of the properdin system presupposes the combined action of properdin, complement, and magnesium ions Chemically pure virus-neutralizing inhibitors do not change in activity after removal of magnesium ions and all other cations by means of prolonged dialysis against frequent changes of distilled water.

(6) Properdin preparations retain their activity at temperatures below −20°C and are rapidly inactivated at +4°C. Virus-neutralizing in-

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(6) Properdin preparations retain their activity at temperatures below −20°C and are rapidly inactivated at +4°C. Virus-neutralizing in-

TABLE II
DIFFERENCES BETWEEN VIRUS-NEUTRALIZING SUBSTANCES AND PROPERDIN OF NORMAL SERA

No	Special properties	Virus-Neutralizing substances	Properdin
1	Content in sera of different animals		
	White rat	Minimal	Maximal
	Guinea pig, rabbit	Maximal	Minimal
2	Biochemical constitution	β-Lipoproteins	Nonhomogeneous, protein (mixed γ-, β-, and α-globulin
3	Fractionation according to Cohn	In fraction III-0	In fraction I
4	Adsorption		
	Zymosan	+	+
	Inulin	−	+
5	Inactivation with trypsin of purified preparation	+	−
6	Comparison or thermal inactivation		
	62°C	One hour	One-quarter hour
	56°C.	Partial after 30 minutes	Fully after 30 minutes
	0–4°C	Stable 4 weeks	Rapid inactivation
	−10°C	Stable over 1.5 years	Requires −20°C and lower
7	Conditions for biological activity		
	Presence of complement:	−	+
	Mg^{++}	−	+
8	Effect of radiation sickness on activity of natural sera	−	+
9	Conditions of removal from natural sera		
	Flexner culture	−	+
	Suspension of influenza virus	+	−

hibitors retain their activity at temperatures of −10°C. for over 18 months, and for some 4 weeks at 4°C.

(7) Chemically pure preparations of thermolabile inhibitors obtained in the form of the β-lipoprotein fraction, freed from bacterial activity in

respect to properdin-sensitive culture of dysentery microorganisms, still possess whole specific virus-neutralizing activity.

On the other hand, the virus-neutralizing activity of properdin preparations, described in a series of papers (Wedgwood *et al*, 1956; Voluiskaya *et al*, 1958, Tovarnitsky, 1959; Ratova *et al*, 1959, Finkelstein *et al*, 1958), is elicited by removal of the properdin by adsorption on zymosan, in the course of which other proteins, including thermolabile, virus-neutralizing inhibitors, are also removed (Polyak *et al*, 1959). For this reason properdin preparations obtained by the method of Pillemer are evidently not homogeneous in respect to their biochemical and immunological composition, but represent a mixture of properdin and virus-neutralizing inhibitors

Table II shows the differential properties of virus-neutralizing inhibitors and properdin

IV. Protective Role of Normal Body Temperature of Animals in Thermal Denaturation of Viruses and Regulation of Their Reproduction

Among protective factors of nonspecific antiviral immunity an important role is played by the effect on the virus of the normal body temperature of warm-blooded animals, as first discovered in the investigations of Smorodintsev *et al* (Smorodintsev and Shishkina, 1948, 1953, Smorodintsev and Zhumatov, 1949, Smorodintsev, 1955) The action of the temperature factor is adverse to the viruses in two distinct ways:

(*1*) A direct inactivation by the normal body temperature of warm-blooded animals (36°–38 5°C) of thermolabile viruses, resulting in a depressant action on most viruses known at present This thermal inactivation exerts its effect both on intracellular and extracellular viruses If the rate of reproduction of the thermolabile virus is less then the rate of its destruction through thermal denaturation, gradual destruction of the virus will occur after a series of passages In this way the resistance of the virus to thermal inactivation will have a pronounced effect on its fate both in the sensitive and in the naturally resistant animal

(*2*) The influence of temperature factors on the process of virus reproduction For most pathogenic viruses the optimal temperature for intracellular reproduction is the physiological body temperature of animals (36°–38 5°C) In the event of a lowering of the physiological temperature, the reproduction of viruses may become selective, and result in the favoring of "cold" variants of viruses, unable to multiply with the usual intensity at the normal body temperature of the animal Strains of viruses arising in the process of their adaptation to the lower

temperature of reproduction can lose all or most of their virulence, and can become attenuated variants (Dubes and Chapin, 1956; Dubes and Wenner, 1957; Lwoff, 1959, 1960; Sabin and Lwoff, 1959).

In the event of a raising of the physiological temperature of reproduction of the viruses to 39°–40°C, highly virulent "thermophilic" variants of the viruses may be selected, with a higher activity of reproduction at the physiological body temperature of animals.

The sensitivity of such viruses to direct inactivation at the temperature of the surroundings may not differ from the thermal sensitivity of the original strain, but on the other hand it may also be significantly lower or higher. In the latter case, the mechanism of attenuation or enhancement of the virulence of "cold" or "thermophilic" strains depends on the sensitivity of the altered variant to the temperature of reproduction and its sensitivity to thermal inactivation.

The experimental temperature conditioning of the poliomyelitis virus with a view to lowering its virulence is particularly important in the context of producing attenuated strains for the production of live vaccine (Lwoff and Lwoff, 1958, 1959; Sabin and Lwoff, 1959).

A. Protective Role of Normal Body Temperature of Warm-Blooded Animals

The protective effect of the normal body temperature of warm-blooded animals, destroying the viruses is made evident by many facts.

(1) The majority of sensitive viruses, pathogenic to animals and man, are distinguished by their high thermolability. When concentrated suspensions of susceptible organs containing maximal concentrations of influenza virus, of tick-borne or Japanese encephalitis, ectromelia, or vaccinia virus are placed in an incubator at 37°C, we observed that after 2–4 days there is a complete loss of biological activity of the infective materials. It should be noted that the period of viability of these viruses in the nonsusceptible organism, when the virus is not in contact with sensitive cells, is of the same order of 2–4 days (Smorodintsev and Shishkina, 1948).

(2) The rate of inactivation of viruses from the brain or subdermal cells, when taken from the infected organism and placed under sterile conditions in the thermostat at 37°C, is in agreement with the data from parallel controls on the rate of inactivation in the same organs of surviving animals. The agreement between the results obtained from parallel experiments on isolated organs and in the live organism is not due to chance, but is to be explained in terms of the same intensity of the effect of temperature on the virus in nonsusceptible tissue.

(3) A change in the body temperature of susceptible and nonsuscepti-

ble organisms has a pronounced effect on the rate of inactivation of viruses introduced. A lowering of the body temperature favors the survival of the virus, while a rise in body temperature hastens its inactivation Thus, the rate of inactivation of influenza virus introduced into the muscle of the hind extremities of white mice or guinea pigs was greatly diminished by artificial cooling, and appreciably speeded up by warming of the infected limb Virus introduced into the peritoneal cavity of frogs or fish, which are particularly suitable for experiments with varying body temperatures, survived for long periods when the animals were in cold water, rapid inactivation of the virus ensued when the frogs and fishes were placed in aquariums with warm water (Smorodintsev and Zhumatov, 1949) (see Table III)

TABLE III
EFFECT OF OUTSIDE TEMPERATURE ON THE INACTIVATION OF INFLUENZA VIRUS INTRODUCED INTO THE PERITONEAL CAVITY OF FROGS

Temperature of aquarium	Virus titers (log 10) in liquid taken from peritoneal cavity of frogs after the indicated number of hours following infection												
	0	4	8	12	24	48	72	96	120	144	168	192	240
1°C	5.5	5.3	4.8	4.8	4.8	3.8	3.8	3.8	3.5	3.3	2.5	1.8	Trace
18°C	5.5	3.3	2.8	2.3	2.8	1.5	0.3	0	0	0	0	0	0
33°–37°C.	5.5	2.8	1.5	0.5	Trace	0	0	0	0	0	0	0	0

(4) Particularly convincing data in favor of the primary role in the mechanism of natural antiviral immunity played by the processes of thermal inactivation of viruses are obtained from observations with viruses having varying initial *in vitro* stability towards temperature factors.

By a process of prolonged adaptation of the influenza virus to unfavorable temperature in developing embryos a thermo-resistant strain was obtained, which was fully developed by prolonged selection of the particles most resistant to temperature While the original strain of influenza virus was destroyed at 52°C during a period of 45–60 minutes, the thermo-resistant strain survived exposure to this temperature for 3 hours.

It was shown that the original strain of influenza virus disappears from the subdermal space of white mice during a period of no more than 3 days, while the thermo-resistant strain takes 6–7 days. There is no biological or antigenic difference between the original and the thermostable variant.

Such thermostable strains are not only more stable in surviving in the

temperature of reproduction can lose all or most of their virulence, and can become attenuated variants (Dubes and Chapin, 1956; Dubes and Wenner, 1957; Lwoff, 1959, 1960; Sabin and Lwoff, 1959).

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The comparison of phage quantities determined after varying intervals following its administration makes it possible to evaluate its dynamics, its distribution, and duration of development in the organism of white mice

The main concentration of all bacteriophages studied is found for a prolonged period of time in those organs and tissues in which fixation of the virus occurs immediately following administration. Thus in subcutaneous administration of phage, the phage persists for a particular period and in particular amounts in the subcutaneous tissue itself

With the majority of bacterial viruses there is a pronounced correlation between the thermal stability of phages in *in vitro* experiments and the duration of their survival in the organism of the animal

Thus, the more thermo-resistant coliphage strain LP is stable in the thermostat for 70 days, and in the organism of white mice it persists for a period of 55 days

Another thermostable phage of *E coli*, strain SP, was preserved *in vitro* at 37°–38°C. for up to 60 days, and for some 40–45 days in mice.

On the other hand, the highly thermolabile *Mycoides* phage is completely inactivated in the thermostat in 6 days, and it disappears from the animal organism after only 4–5 days

A midway position is occupied by coli phages F-1 and F-3, possessing rather higher thermal resistance than *Mycoides* phage, but rather less than coliphages LP and SP The duration of survival of phages F-1 and F-3 in *in vitro* at 37°–38°C averaged 40 days The period of their survival in the organism of mice did not exceed 27–30 days Thus phages with high thermal resistance showed a longer period of persistence in the animal than phages with lesser thermal stability

These results showed a definite correlation between the *in vitro* thermal resistance of isolated bacterial viruses and their persistence in the organism of warm-blooded animals

The effect of thermal resistance of bacterial viruses on their persistence in the organism of warm-blooded animals were also shown in experiments with a strain of *Mycoides* phage, obtained by a process of gradual increase of the temperature acting upon the original thermolabile phage from a culture of *Bacillus mycoides* After 35 passages a strain of bacteriophage was obtained that lysed cultures at 37°C, while the original phage did not lyse cultures at temperatures above 24°–27°C.

At the same time a significant increase in the thermal resistance of the phage occurred· the original strain could endure exposure at 37°C for 6 days, while the figure was 12 days for the altered strain

Comparative experiments to study the persistence of the original and the altered virus in the organism of white mice showed that the original

organism of nonsusceptible animals, but they are also more virulent to susceptible animals

Bacteriophages offer a special and accurate mode for the comparative study of the rates of destruction of viruses in nonsusceptible organisms, and their dependence on thermal stability. They are completely harmless to laboratory animals, but they are nonetheless useful as prototypes of typical viruses, which lend themselves to accurate determination in the organism of animals under test. Since bacteriophages have a rather large span of thermal resistance, it is possible to select both relatively thermolabile and relatively thermostable types for comparative studies of their destruction in warm-blooded animals.

Excellent correlations are observed between the stability of phages to heating and their continued viability in the organism of warm-blooded animals (Petrov, 1953, 1955).

The thermal resistance of phages studied at 56°C varied within fairly wide limits: values varying between 5 minutes and 8 hours were obtained. The viable period of the same phages at temperatures of 37°–38°C. varied between 6 and 70 days

The greatest stability toward the adverse effects of the temperature of the environment was exhibited by some phages of the *Escherichia coli* (LP and SP) and staphylophage types

Average resistance was shown by phages of *E. coli* F-1 and F-3 and *Proteus* phage

There was excellent correlation between experiments carried out at 56°C. and at 37°C. Phages possessing high resistance at a temperature of 56°C. also show high resistance at 37°–38°C.

In order to elicit the relation between the original thermo-resistance of the phages *in vitro* and their prolonged survival in the organism of white mice, a massive subcutaneous or intravenous dose of bacteriophage (10^{10} particles) was administered

Following the subcutaneous administration, the amount of phage was determined at the point of administration, i.e., in the subcutaneous tissue, and in the blood, liver, pancreas, kidney, and urine.

Following intravenous administration, the amount of the phage under study was determined in the blood, liver, kidney, pancreas, lymph nodes, bone marrow, and in the urine following micturition.

In addition, the average amount of phage was measured over the period of observation for the group of mice under observation

Observations were carried out after 15 minutes, 1, 2, 4, 6 hours and subsequently after 1, 3, 6, 9, etc, days after administration of bacteriophage. In separate experiments the observations were continued for up to 70–80 days.

The rate of destruction of viruses in normal tissue emulsions at various temperatures does not follow the relationships established for the behavior of enzymes at different temperatures

Thus a rise in temperature of 10°C increases the rate of destruction of viruses tenfold, while enzymatic reactions are accelerated twofold. The thermal acceleration of cell destruction goes hand in hand with the action of the thermal factor, which, as shown below, can by itself cause spontaneous destruction of viruses without the intervention of enzyme action

When an influenza virus particle loses its biological activity after a short period at a temperature of 37°C, it still retains its immunizing action toward animals and its ability to agglutinate the erythrocytes of chicken and guinea pigs, and also its interference with biologically active viruses when both act on susceptible cells At this stage of biological inactivation the virus micelle is fully susceptible to the action of enzymes, and it rapidly disintegrates, first to relatively large fragments of the original particle, which can still give serological reactions, and subsequently it loses even these antigenic functions

In the light of the data presented, the first stage of inactivation of the virus in unfavorable environments—the loss of its biological activity—takes place without the intervention of tissue enzymes, toward which the normal virus particles possess considerable resistance The first stage of biological inactivation is bound up with the action upon the virus of the normal temperature of warm-blooded animals, and with the action of nonspecific, thermolabile, virus-neutralizing substances of the animal organism

The second stage—the loss by the biologically inactivated virus of its immunogenic and antigenic properties—occurs under the direct action of enzymes, toward which the inactivated influenza virus shows a high degree of susceptibility. For this reason the process occurs much more rapidly in fresh emulsions of various tissues, which are rich in enzymes, unlike inactivated emulsions

The data presented show that the action of the normal body temperature of warm-blooded animals on the viruses, which are present outside susceptible cells, is the immediate cause of their destruction in naturally immune animals

The presence outside the cells of viruses is regularly observed in susceptible animals, when the virus moves from infected cells to normal cells, and also in immunized animals, when the virus loses access to susceptible cells as a result of the action of virus-neutraliz[illegible]

strain persisted in the organism of mice for 4 days, and the altered strain for 11 days (a difference of 7 days), following intravenous administration After subcutaneous administration the original strain persisted for 3–4 days in the subcutaneous space, and the altered strain for a period of 10 days (Petrov, 1953) (see Table IV).

TABLE IV
PERIOD OF PERSISTENCE OF BACTERIOPHAGES AFTER ADMINISTRATION IN DIFFERENT TISSUES*

	Intravenous			Subdermal		
Bacteriophage	General tissue of mice	Pancreas	Lymphatic nodes	General tissue of mice	Pancreas	Lymphatic nodes
Original thermolabile phage strain	4	4	4	3	3	3
Thermostable variant of the same strain	11	10	11	10	9	9

*In days

In respect to all other criteria the thermostable strain is indistinguishable from the original strain. Thus the mere increase in the thermal stability of the phage resulted in a significant lengthening of the period of its survival in the organism of warm-blooded animals.

These investigations demonstrate that the normal body temperature of warm-blooded animals plays an important protective role, constituting an important physiological factor that leads to spontaneous destruction of viruses situated inside and outside the susceptible cells of the animal organism.

The clear correlation between the intensity of destruction of the virus in the naturally resistant organism and the body temperature cannot be explained in terms of fermentative processes which are accelerated by an increase in the temperature of the surroundings. The data in existence testify to the inertness of the enzymes (both in the tissue and also in purified form) in respect to the influenza virus and others. For this reason one cannot attach too much importance to the role of enzymes in destroying the biological activity of viruses (Smorodinstev and Shishkina, 1953).

For example, our investigations show that the destruction of viruses takes place at the same rate with influenza virus at 37°C. in normal tissue emulsions of chick embryos or of internal organs as it does in tissue emulsions of the same organs which had previously been inactivated at 90°–100°C.

The rate of destruction of viruses in normal tissue emulsions at various temperatures does not follow the relationships established for the behavior of enzymes at different temperatures

Thus a rise in temperature of 10°C increases the rate of destruction of viruses tenfold, while enzymatic reactions are accelerated twofold The thermal acceleration of cell destruction goes hand in hand with the action of the thermal factor, which, as shown below, can by itself cause spontaneous destruction of viruses without the intervention of enzyme action.

When an influenza virus particle loses its biological activity after a short period at a temperature of 37°C, it still retains its immunizing action toward animals and its ability to agglutinate the erythrocytes of chicken and guinea pigs, and also its interference with biologically active viruses when both act on susceptible cells At this stage of biological inactivation the virus micelle is fully susceptible to the action of enzymes, and it rapidly disintegrates, first to relatively large fragments of the original particle, which can still give serological reactions, and subsequently it loses even these antigenic functions

In the light of the data presented, the first stage of inactivation of the virus in unfavorable environments—the loss of its biological activity—takes place without the intervention of tissue enzymes, toward which the normal virus particles possess considerable resistance The first stage of biological inactivation is bound up with the action upon the virus of the normal temperature of warm-blooded animals, and with the action of nonspecific, thermolabile, virus-neutralizing substances of the animal organism

The second stage—the loss by the biologically inactivated virus of its immunogenic and antigenic properties—occurs under the direct action of enzymes, toward which the inactivated influenza virus shows a high degree of susceptibility For this reason the process occurs much more rapidly in fresh emulsions of various tissues, which are rich in enzymes, unlike inactivated emulsions

The data presented show that the action of the normal body temperature of warm-blooded animals on the viruses, which are present outside susceptible cells, is the immediate cause of their destruction in naturally immune animals.

The presence outside the cells of viruses is regularly observed in susceptible animals, when the virus moves from infected cells to normal cells, and also in immunized animals, when the virus loses access to susceptible cells as a result of the action of virus-neutralizing antibodies

When assessing the behavior of viruses with different thermal sensitivities in the organism of susceptible animals, only limited weight must

be given to this factor as an indication of its virulence. A greater or lesser thermal resistance of the virus does not necessarily account for the reproductive activity or the interaction of the virus with the sensitive organism. Many examples can be found where the virus is less pathogenic to susceptible animals, despite its high thermal resistance, than a thermolabile strain of a homologous type, but of a different origin On the other hand, there is little doubt that one and the same virus, with a certain virulence for a given type of animal, can be altered, although not very commonly, to a more or less virulent variant by a process of raising or lowering its stability toward thermal denaturation. In our laboratory this has been achieved with viruses of influenza and ectromelia. An increase in thermal resistance of these viruses toward heating at 50°–56°C. by a prolonged process of selecting thermo-resistant variants was accompanied by a corresponding increase in their virulence for white mice if the observed variant lost its ability to be reproduced at lower temperature. Major changes in virulence (either in the direction of increased or decreased virulence) can be observed in the production of experimental variants adapted to a higher or lower temperature of reproduction in susceptible tissues, e g, in tissue cultures.

A great deal of experimental material has been accumulated on the subject of thermal stability of attenuated variants of poliomyelitis virus, which can be distinguished by this property from pathogenic strains (Lwoff and Lwoff, 1958, 1959; Sabin and Lwoff, 1959).

The nature of the effects of temperature (T) in inactivating infectious particles of poliovirus or in disturbing the capacity for reproduction at different temperatures (RCT) are not fully understood. The RCT relationship is only one of the factors controlling neurovirulence (Lwoff, 1960).

The justification of a scientific theory lies in its ability to explain observable facts and especially in its ability to predict them. Our concept of the importance of the process of thermal inactivation of viruses and its protective role in nonspecific resistance to viruses and of the neutralization of their biological activity by the thermolabile inhibitors of normal serum is confirmed by the ability to calculate with fair accuracy the time of destruction of any virus in the organism of nonsusceptible animals on the basis of the following measures of the biological stability of the virus: (*1*) Resistance to unfavorable temperature (37°C., 52°C.) in an *in vitro* experiment; and (*2*) resistance to the action of thermolabile substances of the normal serum of the animal under test (see Table V).

A virus with a high order of resistance to both factors will survive for prolonged periods in a viable state in the nonsusceptible organism. A

TABLE V

CORRELATION BETWEEN THERMAL STABILITY, THEIR SENSITIVITY TO VIRUS-NEUTRALIZING INHIBITORS OF NORMAL SERUM *in Vitro*, AND THEIR PERSISTENCE IN THE ORGANISM OF WHITE MICE *in Vivo*[a]

Phage strain under examination	Thermal stability *in vitro* at the indicated temperature			Sensitivity to the action of virus-neutralizing inhibitors or normal serum		Theoretical evaluation of persistence in the organism of white mice	Actual period of phage survival in the organism of white mice (days)
	56°C (hours)	37°C (days)	The level of thermal resistance	Period of survival in contact with fresh serum of normal mice	Index of resistance		
Coliphage LP	8.0	70	High	12 days	High	Very high	60
Coliphage SP	6.0	60	High	12 days	High	Very high	50
Coliphage F-1	3.5	45	Medium	12 days	High	High	30
Mycoides phages F-3	0.1	6	Low	3 days	Medium	Short	4
Staphylococcus phage	4.0	50	Medium	1–3 hours	Low	Short	4
Proteus phage	3.0	30	Medium	1 day	Low	Short	6

[a] Following subcutaneous or intravenous inoculation

be given to this factor as an indication of its virulence. A greater or lesser thermal resistance of the virus does not necessarily account for the reproductive activity or the interaction of the virus with the sensitive organism Many examples can be found where the virus is less pathogenic to susceptible animals, despite its high thermal resistance, than a thermolabile strain of a homologous type, but of a different origin. On the other hand, there is little doubt that one and the same virus, with a certain virulence for a given type of animal, can be altered, although not very commonly, to a more or less virulent variant by a process of raising or lowering its stability toward thermal denaturation. In our laboratory this has been achieved with viruses of influenza and ectromelia An increase in thermal resistance of these viruses toward heating at 50°–56°C by *a prolonged process of selecting thermo-resistant* variants was accompanied by a corresponding increase in their virulence for white mice if the observed variant lost its ability to be reproduced at lower temperature Major changes in virulence (either in the direction of increased or decreased virulence) can be observed in the production of experimental variants adapted to a higher or lower temperature of reproduction in susceptible tissues, e g, in tissue cultures.

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A virus with a high order of resistance to both factors will survive for prolonged periods in a viable state in the nonsusceptible organism A

TABLE VI

EFFECT OF ADAPTATION OF INFLUENZA VIRUS TYPES A AND A_1 OF RELATIVELY LOW VIRULENCE IN MICE TO INCREASED TEMPERATURES OF SURVIVAL (*in Vitro*) OR OF REPRODUCTION (*in Vivo*) ON ITS VIRULENCE IN MICE

Type of influenza virus	Adaptation conditions	Strains	Index of thermal resistance (days of survival at 37°C) *in vitro*	Maximum virus concentrations (log 10) in developing chick embryos at		LD_{50} for white mice (log 10)		Virulence
				32.0°C	48.5°C	Expt I	Expt II	
A_1	*In vitro*	Original	3	8.2	6.2	6.6	6.2	Increased
		Altered	8	6.1	7.4	4.2	3.9	
A_1	*In vivo*	Original	3	8.2	6.2	6.6	6.3	Increased
	(developing chick embryo, 40°C)	Altered	8	3.6	8.2	2.4	2.1	
A	*In vivo*	Original	3	7.0	7.4	4.8	5.1	Decreased
	(developing chick embryo, 32°C)	Altered	2	8.2	5.6	>7.0	>7.2	
A	*In vivo*	Original	3	7.0	7.4	4.8	5.0	Not changed
	(developing chick embryo, 32°C)	Altered	3	8.2	7.6	4.9	4.9	

virus having a low order of resistance to both factors, or to one of them, will be destroyed rapidly, and its period of survival in the nonsusceptible organism will be minimal (Smorodintsev, 1957b).

The protective role of the thermal factor and of the virus-neutralizing inhibitors is also confirmed by the study of the mechanism of nonspecific resistance in the rather more complex conditions prevailing in *the susceptible organism, where the intensity of their interaction with* the virus depends largely on the overriding factor of the degree of susceptibility to the virus of the sensitive cells

For this reason the rate of multiplication of various strains of pathogenic viruses in the susceptible organism assumes a major role in determining the sensitivity of its cells to a given virus under concrete conditions of development of the infective process. The fate of the virus in the organism with specific immunity will depend primarily on specific factors of defense rather than on nonspecific mechanisms. Despite the complexity of the problem concerning the prognosis of the developing virus infection, it is possible to operate with relative indications of *amelioration or intensification of the course of virus infection* produced by a given strain, when the following properties are altered: *(1)* The temperature optimum of reproduction of the virus [Sabin's RCT (1959), or reproductive capacity at different temperatures]; *(2)* the index of thermal inactivation of the virus at 37°C.; and *(3)* the sensitivity to the action of thermolabile inhibitors. As in the experiments with nonsusceptible animals, it is possible to affect the behavior of the virus in susceptible tissue by artificially changing its response to temperature of reproduction, its resistance to thermal denaturation, and its sensitivity to virus-neutralizing inhibitors An increased stability toward these factors may accelerate the rate of multiplication and raise the virulence of the virus, while lessened stability favors the production of less virulent viruses (see Table VI)

The further study of the protective role of normal body temperature on the development of virus infections in susceptible warm-blooded animals and of the significance of the principle of adaptation of viruses to a lowering of the temperature of reproduction are essential for the experimental preparation of attenuated vaccine strains.

On the basis of our experience, the attenuation of viruses by selection of strains adapted to the reproduction by low temperature (25°–32°C.) is possible only for variants which have lost simultaneously their original ability to multiply at 39°–40°C. If this higher temperature level of reproduction is not changed but remains the same in the course of prolonged development at low temperature, no attenuation occurs. We

TABLE VI

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Type of influenza virus	Adaptation conditions	Strains	Index of thermal resistance (days of survival at 37°C) *in vitro*	Maximum virus concentrations (log 10) in developing chick embryos at		LD_{50} for white mice (log 10)		Virulence
				32.0°C	43.5°C	Expt I	Expt II	
A_1	*In vitro*	Original	3	82	62	66	62	Increased
		Altered	8	61	74	42	39	
A_1	*In vivo*	Original	3	82	62	66	62	Increased
	(developing chick embryo, 40°C)	Altered	6	36	82	24	21	
A	*In vivo*	Original	3	70	74	48	51	Decreased
	(developing chick embryo, 32°C)	Altered	2	82	56	>70	>72	
A	*In vivo*	Original	3	70	74	48	50	Not changed
	(developing chick embryo, 32°C)	Altered	3	82	76	49	49	

virus having a low order of resistance to both factors, or to one of them, will be destroyed rapidly, and its period of survival in the nonsusceptible organism will be minimal (Smorodintsev, 1957b).

The protective role of the thermal factor and of the virus-neutralizing inhibitors is also confirmed by the study of the mechanism of nonspecific resistance in the rather more complex conditions prevailing in the susceptible organism, where the intensity of their interaction with the virus depends largely on the overriding factor of the degree of susceptibility to the virus of the sensitive cells.

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feron results from irradiation of the virus with UV light Influenza virus inactivated with 0 02% formalin loses its power of interference and of producing interferon The production of interferon does not take place if the cell is at the same time partaking in the synthesis of virus For this reason the simultaneous introduction of living and dead virus does not stimulate the production of interferon, for which it is necessary to administer the dead virus several hours prior to infection; this point is also important in eliciting interference

Sendai virus obtained from calf's kidney tissue cultures stimulates the production of interferon after a 24-hour period of growth, which appears during a period when the rate of reproduction of the virus is falling The same result is obtained under conditions of intense multiplication of influenza virus on chorioallantoic membrane of growing chick embryos During the first 24 hours the multiplication of virus approximates to its peak value, but no interference is observed, as the reproduction of the virus slackens, so the production of interferon starts

Burke and Isaacs (1958) suppose that in the case discussed the production of interferon is evoked by the virus, which is spontaneously inactivated after a period of 24 hours at 37°C, and that interferon is not elaborated if the cells are fully integrated into the production of virus

Tissue cultures chronically infected with Newcastle virus and other viruses, and which retain their normal morphological structure, develop resistance to other viruses possessing cytopathogenic activity The resistance is connected with the production of an analogous interferon substance, which can be inactivated with trypsin, which is not adsorbed by erythrocytes, which does not enter into contact with antibodies, and which does separate after centrifugation at 25,000 *g* (Henle *et al*, 1959).

Ho and Enders (1959a,b) observed the production of an inhibiting substance of the interferon type in a culture of human kidney cells infected with an avirulent strain of poliomyelitis virus Type 2, which militated against the infection of the cells of human kidney and amnion by the same or other type of poliomyelitis virus, or by other viruses

B Elimination of Elementary Bodies and Virus Inclusions

A different type of protective process is represented by the reaction of the infected cell directed at re-establishing normal metabolism by freeing the cell interior from the foreign virus particles by a process of mechanical separation from the infected cell The elimination of elementary bodies in this case assists the establishment of physiological function and restricts the dissipation of natural synthetic resources on the reproduction of the parasitic agent, and also its toxic activity on

have repeatedly observed such results for strains of the adenovirus group, influenza, and tick-borne encephalitis viruses.

V. Protective Reactions of Susceptible Cells

In analyzing the various mechanisms of protective reactions mobilized by the infected cells, with the result of counteracting the multiplication of the virus and minimizing the danger of consequent disorganization of the metabolism and physiological function of the cell, two factors which have received considerable study deserve special mention.

A Production of Interferon

In studying the interference, developing in the chorioallantoic membrane under the influence of influenza virus inactivated by heating, the appearance of a substance was observed interfering with the virus; its physicochemical and antigenic properties differed from those of the inactivated virus (Isaacs and Lindenmann, 1957). Since the substance discovered was connected with the process of interference, it was named *interferon.*

It appeared that interferon inhibited the multiplication of a wide range of viruses, belonging to the group of myxoviruses, smallpox, and cowpox viruses. Interferon does not exhibit a greater activity toward a homologous virus than a heterologous virus. Prolonged interaction with the virus at 37°C, and to a lesser extent at 2°C, accentuates the action of interferon. Interferon does not act directly on the virus, and does not affect its adsorption by the cell, but retards virus multiplication in susceptible tissues, especially in those homologous in relation to the formation of the interferon The quantity of interferon formed in the cell is increased by repeated contact with the live virus. One supposes that under the influence of inactivated virus intermediate products of normal *virus particle synthesis are formed. Such a cell, interacting with* the active virus, produces added quantities of interferon, which deflects the cells from direct synthesis of virus particles In the light of this theory, interference represents a deviation of normal virus synthesis with the production of interferon

It is not as yet known whether interferon represents a single substance or a group of similar substances The activity of viruses stimulating the production of interferon appears proportional to its interference activity. A certain production of interferon is elicited by influenza virus which has been inactivated by heating for 1 hour at 56°C. or has been incubated at 37°C. for a prolonged period, whereas inactivation at 60°C. destroys its power of interference and also its ability to produce interferon. A high interference activity as well as high production of inter-

formed into morphologically distinct intracellular inclusions (Pigarevsky, 1953, 1958) This spontaneous appearance of a nonspecific cellular resistance is capable of fairly precise virological and morphological control, and is characterized by a definite correlation with the virulence of the virus and the general resistance of the susceptible organism (Luzyanina, 1957; Al A Smorodintsev, 1957; Smorodintsev and Luzyanina, 1958) We studied this problem using as a model white mice experimentally infected with influenza-type infections, employing variants with high and low virulence of the same strain of A_1 influenza virus The regular formation of characteristic basophilic inclusions, (previously described by Loosli, 1949, Harford and Hamlin, 1952, Harford *et al*, 1955, Hoyle, 1954, Pigarevsky, 1953, 1957a,b) in the altered cells of the epithelium of the lower respiratory tract were first observed The basophilic inclusions represent colonies of virus, and under the electron microscope the presence of many elementary bodies of influenza virus can be determined They have a rounded oval or sickled shape, are colored by Feulgen's reagent, are present in varying amounts in the cell cytoplasm, and do not have a membrane Their presence in large amounts is a sign of the virulent course of the influenza infection

Microcolonies of influenza virus can be present in different parts of the cytoplasm of one and the same cell In the case of diffuse invasion of the cytoplasm by the virus, the cell is undergoing degeneration In the apical strata of the cytoplasm one can observe the detaching of infected portions of the cytoplasm, which preserve the cell from destruction The detachment of infected portions of cytoplasm can be considered as a manifestation of a protective process on the part of the cell, directed at removal of the noxious factor, after which the cell is regenerated and recovers

When the course of the influenza is benign, as happens soon after infecting mice with a strain of low virulence, the formation of many inclusions of a different character are observed at the same time as the basophilic bodies, of a type which stain with acid dyes (oxyphil inclusions), and which take the form of relatively large objects of 0 5–5 5μ in diameter in the cell cytoplasm In contradistinction to the basophilic inclusions [illegible]

[illegible]

[illegible] particles from direct contact with the cytoplasm.

The relationship between the quantities of oxyphilic and basophilic inclusions provides an indication of the severity of the influenza infection A preponderance of oxyphilic inclusions points to a benign course,

the internal structures of the cell. Such a form of benign interaction of the virus and the cells appears to be quite frequent and typical in the majority of known virus infections, which but rarely result in the partial or total destruction and death of the cells to be seen under the influence of a range of cytopathogenic viruses.

The separation of virus particles from the cell occurs by various means: the one most commonly observed appears to be a continuous process of elimination from the cell of elementary virus bodies formed prior to establishment of the infective virus in the cytoplasm or nucleus of the cell The study of the reproduction of a series of viruses, devoid of pathogenic activity, in single layer cultures of tissue confirms the rapid elimination of particles accumulated in the cells in the nutrient medium, together with complete conservation of the normal morphological structure of the infected cells. Under these conditions, as also in experiments with isolated cells, it is evident that large masses of elementary bodies can pass through the normal morphological barrier of the cell wall. The mechanism of this elimination is not very clear as yet. It is possible that the passage of the virus is preconditioned by fermentative cleavage of the mucoproteins and other components of the cell wall, giving rise to microscopic pores ready for the re-exit of the virus particles from the confines of the cell It is possible that the elementary particles first arise from the semiliquid cytoplasm adjacent to the cell wall, and are then mechanically pressed through the elastic cell membrane by virtue of their high internal turgidity; the membrane then closes again, rather after the fashion of a rubber stopper which has been punctured by a hypodermic needle.

Electron microscopic study of cells infected with influenza type A_1 virus, or with hem-adsorbing parainfluenza viruses, confirms the presence of free elementary bodies on the outer wall of the cell in the form of threadlike structures issuing from the cell membrane, and firmly attached to it. In this case one is evidently dealing with physical growth of the virus through the membrane, arising from the peripheral regions of the cytoplasm, in which the elementary bodies are liberated into the surrounding medium as from a porous mold, filled with chains of adhering virus particles The phenomenon of adsorption of erythrocytes on the surface of the cell is peculiar to this group of virus infections, in which the virus being extruded continues to maintain a firm adherence to the cell membrane, and under these conditions it carries the massive weight of the adsorbed erythrocyte.

Another form of elimination of virus particles is frequently observed, which proceeds by the elimination outside the cell barrier of groups of elementary bodies accumulated in the colonies of virus, previously

Thus the protective processes of elimination on the part of the infected cell proceed via the following processes. (*1*) Separation of the accumulating elementary bodies in the semi-liquid cytoplasm and their elimination by various means from the interior of the infected cell, (*2*) isolation of the virus particles into separate colonies, where they are surrounded with a reactive membrane which is impermeable to the virus, (*3*) evacuation of the inclusions outside the infected cell, where they undergo phagocytosis and isolation in the interior of leucocytes from contact with fresh susceptible cells.

According to the data of Zilber *et al* (1937, 1946), the processes of elimination of viruses through the kidneys play an important part in the mechanism of natural nonspecific antiviral immunity Viruses which enter into the organism of naturally resistant or immunized animals circulate freely in the blood and organs if they have no affinity for their cells, whence they are continuously excreted in the urine through the kidneys. Animal immunity to ultraviral agents is always accompanied by high concentrations of virus in the kidneys, and their elimination in the urine

In susceptible animals the viruses, according to Zilber, undergo rapid fixation in the sensitive organs and show no tendency to enter the general circulation with subsequent elimination through the kidneys.

Confirmatory experiments have failed to substantiate the findings on which the above described view of the role of excretory mechanism via the kidney in antiviral immunity by L A Zilber was based (Zhumatov, 1955c; Smorodintsev, 1955, Petrov, 1955).

In the organism of susceptible animals the virus is not only fixed in susceptible tissues (which would support L A Zilber), but also multiplies within them, causing a severe infection in the vascular systems and the internal organs *In this context it is found that in susceptible* animals the pathogenic viruses are found more frequently and in higher concentration at the point of entry, then to be dissipated into the general circulation whence they reach the kidneys, traveling through the damaged vascular system into the urine However, even in susceptible animals the amount of virus which is found in the urine is quite negligible compared with the massive concentrations at the points of reproduction in the infected organism

In the naturally nonsusceptible or immunized organism, on the other hand, there is a negligible spread of the virus from the local focus. The virus corpuscles are poorly resorbed from the point of their initial localization, so that the mechanisms of separation through the kidney with consequent evacuation of the virus from the organism are quite negligible Virus is less regularly observed in the urine of naturally

while a preponderance of basophilic inclusions points to a severe course of the experimental influenza infection.

If the influenzal infection is produced in mice which have previously been exposed to 400–800 r of X-ray irradiation, then simultaneously with the appearance of radiation sickness, a more severe course is taken by the infective process, and the quantity of oxyphilic inclusions is reduced by several times in relation to infections in mice which have not previously been irradiated (Al. A. Smorodintsev, 1957).

In infections of white rats, which are resistant to experimental influenza, with a strain of virus that is virulent for mice, a markedly higher amount of oxyphilic (fuchsinophilic) inclusions are formed than in the white mice

There is an obvious correlation between the amount of oxyphilic inclusions formed and the severity of the influenzal infection. The absence of these reactive manifestations is accompanied by intensive damage to the epithelium of the trachea, bronchi, and bronchioles in susceptible animals

Analogous correlations between the presence in susceptible tissue of oxyphilic cytoplasmic inclusions, morphologically isolated from the cell, and the severity of the virus infection are observed with a series of other diseases, e g., experimental rabies Negri bodies are readily formed in the initial period of adaptation of the naturally occurring virus in the brain of rabbits and white mice, but they are not to be observed in the examination of highly virulent strains after its virulence is stabilized. The formation of cytoplasmic inclusions provides an index of a high level of innate resistance of the cell, and its reactive potential in relation to the isolation and subsequent elimination of the intracellular parasite

The oxyphilic influenza virus inclusions found in epithelial cytoplasm are regularly to be found outside the cell boundary in the lumen of the trachea and bronchi. The possibility of elimination from the cell of inclusions having a diameter of up to 6μ is an indication of the high degree of permeability of the cell membrane for foreign bodies of considerable magnitude, which occurs without apparent damage, and helps in the repair and regeneration of infected tissue.

The virus inclusions eliminated into the lumen of the trachea, bronchi, and bronchioles undergo intense phagocytosis, facilitated by the further isolation of colonial aggregates of virus particles in the interior of the leucocytes. Under these conditions we have not been able to observe the liberation of the virus from the inclusion membranes in view of the high stability to tissue enzymes. But the active virus occluded in the interior of the membranes rapidly dies as a result of thermal denaturation

Thus the protective processes of elimination on the part of the infected cell proceed via the following processes: (1) Separation of the accumulating elementary bodies in the semi-liquid cytoplasm and their elimination by various means from the interior of the infected cell; (2) isolation of the virus particles into separate colonies, where they are surrounded with a reactive membrane which is impermeable to the virus, (3) evacuation of the inclusions outside the infected cell, where they undergo phagocytosis and isolation in the interior of leucocytes from contact with fresh susceptible cells.

According to the data of Zilber *et al.* (1937, 1946), the processes of elimination of viruses through the kidneys play an important part in the mechanism of natural nonspecific antiviral immunity. Viruses which enter into the organism of naturally resistant or immunized animals circulate freely in the blood and organs if they have no affinity for their cells, whence they are continuously excreted in the urine through the kidneys. Animal immunity to ultraviral agents is always accompanied by high concentrations of virus in the kidneys, and their elimination in the urine.

In susceptible animals the viruses, according to Zilber, undergo rapid fixation in the sensitive organs and show no tendency to enter the general circulation with subsequent elimination through the kidneys.

Confirmatory experiments have failed to substantiate the findings on which the above described view of the role of excretory mechanism via the kidney in antiviral immunity by L A Zilber was based (Zhumatov, 1955c; Smorodintsev, 1955; Petrov, 1955).

In the organism of susceptible animals the virus is not only fixed in susceptible tissues (which would support L A Zilber), but also multiplies within them, causing a severe infection in the vascular systems and the internal organs. In this context it is found that in susceptible animals the pathogenic viruses are found more frequently and in higher concentration at the point of entry, then to be dissipated into the general circulation whence they reach the kidneys, traveling through the damaged vascular system into the urine However, even in susceptible animals the amount of virus which is found in the urine is quite negligible compared with the massive concentrations at the points of reproduction in the infected organism

In the naturally nonsusceptible or immunized organism, on the other hand, there is a negligible spread of the virus from the local focus. The virus corpuscles are poorly resorbed from the point of their initial localization, so that the mechanisms of separation through the kidney with consequent evacuation of the virus from the organism are quite negligible. Virus is less regularly observed in the urine of naturally

while a preponderance of basophilic inclusions points to a severe course of the experimental influenza infection.

If the influenzal infection is produced in mice which have previously been exposed to 400–800 r of X-ray irradiation, then simultaneously with the appearance of radiation sickness, a more severe course is taken by the infective process, and the quantity of oxyphilic inclusions is reduced by several times in relation to infections in mice which have not previously been irradiated (Al A. Smorodintsev, 1957).

In infections of white rats, which are resistant to experimental influenza, with a strain of virus that is virulent for mice, a markedly higher amount of oxyphilic (fuchsinophilic) inclusions are formed than in the white mice

There is an obvious correlation between the amount of oxyphilic inclusions formed and the severity of the influenzal infection. The absence of these reactive manifestations is accompanied by intensive damage to the epithelium of the trachea, bronchi, and bronchioles in susceptible animals.

Analogous correlations between the presence in susceptible tissue of oxyphilic cytoplasmic inclusions, morphologically isolated from the cell, and the severity of the virus infection are observed with a series of other diseases, e g., experimental rabies Negri bodies are readily formed in the initial period of adaptation of the naturally occurring virus in the brain of rabbits and white mice, but they are not to be observed in the examination of highly virulent strains after its virulence is stabilized. The formation of cytoplasmic inclusions provides an index of a high level of innate resistance of the cell, and its reactive potential in relation to the isolation and subsequent elimination of the intracellular parasite

The oxyphilic influenza virus inclusions found in epithelial cytoplasm are regularly to be found outside the cell boundary in the lumen of the trachea and bronchi. The possibility of elimination from the cell of inclusions having a diameter of up to 6μ is an indication of the high degree of permeability of the cell membrane for foreign bodies of considerable magnitude, which occurs without apparent damage, and helps in the repair and regeneration of infected tissue.

The virus inclusions eliminated into the lumen of the trachea, bronchi, and bronchioles undergo intense phagocytosis, facilitated by the further isolation of colonial aggregates of virus particles in the interior of the leucocytes. Under these conditions we have not been able to observe the liberation of the virus from the inclusion membranes in view of the high stability to tissue enzymes. But the active virus occluded in the interior of the membranes rapidly dies as a result of thermal denaturation.

Thus the protective processes of elimination on the part of the infected cell proceed via the following processes: (*1*) Separation of the accumulating elementary bodies in the semi-liquid cytoplasm and their elimination by various means from the interior of the infected cell; (*2*) isolation of the virus particles into separate colonies, where they are surrounded with a reactive membrane which is impermeable to the virus, (*3*) evacuation of the inclusions outside the infected cell, where they undergo phagocytosis and isolation in the interior of leucocytes from contact with fresh susceptible cells.

According to the data of Zilber *et al* (1937, 1946), the processes of elimination of viruses through the kidneys play an important part in the mechanism of natural nonspecific antiviral immunity Viruses which enter into the organism of naturally resistant or immunized animals circulate freely in the blood and organs if they have no affinity for their cells, whence they are continuously excreted in the urine through the kidneys. Animal immunity to ultraviral agents is always accompanied by high concentrations of virus in the kidneys, and their elimination in the urine.

In susceptible animals the viruses, according to Zilber, undergo rapid fixation in the sensitive organs and show no tendency to enter the general circulation with subsequent elimination through the kidneys.

Confirmatory experiments have failed to substantiate the findings on which the above described view of the role of excretory mechanism via the kidney in antiviral immunity by L A. Zilber was based (Zhumatov, 1955c; Smorodintsev, 1955, Petrov, 1955)

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In the naturally nonsusceptible or immunized organism, on the other hand, there is a negligible spread of the virus from the local focus The virus corpuscles are poorly resorbed from the point of their initial localization, so that the mechanisms of separation through the kidney with consequent evacuation of the virus from the organism are quite negligible. Virus is less regularly observed in the urine of naturally

resistant animals, and in significantly lower amounts than observed in susceptible animals.

Only when the virus is administered intravenously to resistant animals, together with toxic virus and tissue material, is the transfer into the urine of virus particles sharply increased, accompanied by acute damage to the vascular system of the kidney. This can be observed, for example, when bacteriophage is administered into the veins of white mice together with a lysed culture of one or other of the microbes toxic for the animal. Under these conditions the phage passes the vascular barrier of the kidneys more easily. At the same time the normal vascular barriers of the kidneys are scarcely permeable either for the virus or even for much smaller protein molecules.

The quantitative aspect of the passage of viruses through the excretory organs of animals with natural immunity are characterized by negligible factors, representing thousandths of a per cent for large virus masses, which undergo destruction in the organism of nonsusceptible animals

It is therefore incorrect to think of the passage of one or other of the viruses through the glomerular filter, the alimentary canal, the mammary gland, etc., as falling into the category of protective mechanisms. Thus, the elimination in the urine of enteric typhoid organisms, streptococci, tularemia agents, etc, is always indicative of serious damage to the vascular system of the kidneys, and cannot therefore be regarded as a protective factor. Similarly the passage of virus into the urine, milk, and intestinal contents in infections of susceptible or nonsusceptible animals signals the development of vascular infection, which evidently is not an "immunity factor" but a pathological symptom of a particular process, of interest more to the pathologist and epidemiologist rather than to the immunologist (Smorodintsev, 1957a).

VI. Role of Phagocytic Factors in the Destruction of Animal Viruses

The well-authenticated protective role of phagocytic factors in natural and acquired bacterial immunity has for many years been accepted also in the case of virus infections of animals. It was supposed that the leucocytes of normal or immunized animals are able to produce phagocytosis of virus particles and assist in the inactivation (Fairbrother, 1933; Jamuni and Holden, 1934). However, this was not confirmed in the investigations of Sabin (1935), Zilber *et al.* (1937), and Smorodintsev *et al* (Smorodintsev and Shishkina, 1940, 1942; Smorodintsev, 1955; Zhumatov, 1955b)

We have carried out a systematic study of the role of phagocytic

factors in the destruction of viruses, using a number of different methods.

One of the simplest, although also one of the less exact models for phagocytic experiments, is represented by a suspension of polymorphonuclear leucocytes or monocytes which have been washed free of serum, or have been suspended in a solution of normal serum The results of their interaction with viruses depend in large measure on the method of preparation of the virus suspensions Viruses which have been freed thoroughly from tissue fragments and made into a homogeneous mixture do not undergo phagocytosis even in the presence of antibodies and complement, whereas viruses contained in fragments of comminuted infected tissue are easily attacked by the leucocytes Under these conditions a digestion of the tissue fragments occurs, generally without affecting the biological activity of the virus, which enters into the interior of the leucocytes, together with tissue fragments

Experiments with isolated leucocytes are best carried out, not under artificial conditions, but under the natural conditions of the organism, using for this purpose the direct observation of the interaction of viruses with exudate from leucocytes, which is taken within the peritoneal cavity a few hours following the intraperitoneal introduction of a 10% solution of peptone broth or aleuronate in nutrient broth During the first few hours after the preparation, the peritoneal cavity of white mice, white rats, or guinea pigs is filled with polymorphonuclear leucocytes, and becomes suitable for carrying out phagocytosis experiments with various microorganisms When 100 to 1000 lethal doses of cultures of a given virulence of streptococci, staphylococci, or *Salmonella* bacilli are introduced into animals with mobilized leucocytic exudate, the infective process is rapidly terminated, due to the prior mobilization of the phagocytic protective factors. Under similar conditions of action of natural leucocytic exudate on viral agents, the phagocytic activity does not result in inactivation of most of the virus species studied by us

In experiments on the phagocytosis of viral agents, we do not recommend working with pure, homogeneous elementary bodies, which like other proteins undergo phagocytosis to a negligible degree, on account of their very small size This is confirmed by the low infectivity of leucocytic exudate taken from the peritoneal cavity 15, 30, 60, and 120 minutes after infection and freed from adsorbed virus particles with homologous antiserum, followed by washing up and ultrasonic or electrical homogenizing Only the rather larger viruses of the psittacosis-mouse pneumonia group are an exception to this and are susceptible to phagocytosis, while at the same time the large viruses of smallpox-vaccinia and ectromelia are not subject to phagocytic action (Meyer, 1941; Zhumatov, 1955b; Drobyshevskaya, 1959). An unusual type of

phagocytosis is represented by some viruses like those responsible for lymphocytic choriomeningitis, for which leucocytes represents a susceptible type of cell The presence of large amounts of such viruses in the leucocytic exudate arises not as a result of phagocytosis, but of the multiplication of the virus in the susceptible leucocytes (Medvedkova, 1955; see also Merling, 1945)

Bearing in mind the fact that in the organism of infected animals the virus exists not only in the form of free elementary particles, but frequently is also adsorbed on the surface of erythrocytes, of fragments of disintegrated tissue, and even more frequently as inclusions in fragments of disintegrating infected cells, we have developed a convenient method of carrying out phagocytosis experiments, involving the certainty of substantial penetration of significant amounts of virus into the interior of leucocytes To do this, a suspension of erythrocytes, on the surface of which the type of virus under study had been adsorbed (Zhumatov, 1955b), or otherwise fragments of infected tissue (brain, lungs, spleen tissue culture) comminuted to 3–7μ and washed free from virus particles, was introduced into the peritoneal cavity preconditioned to produce leucocytotic exudate. After 15, 30, and 60 minutes the gradual increase in phagocytosis of tissue material (nucleated erythrocytes, tissue fragments) was followed in stained smears of the exudate, and was shown to reach the stage of complete intracellular digestion after a period of 2–4 hours, showing a high degree of phagocyte activity in the animals observed A check on the virus content of the leucocytes from the exudate under study shows a high degree of infectivity of the leucocyte suspension at the period of complete involvement of the tissue fragments However, this high state of virus activity remains after 4–6 hours, when the tissue material has been fully digested in the cytoplasm of the leucocytes

The results of our observations, summarized in Table VII, confirm the incomplete extent of phagocytosis, the inability of phagocytosis to inactivate the majority of the viruses studied, with the exception of the rather larger viruses of the psittacosis-pneumonia (white mice) group.

The intensive phagocytosis of virus particles combined with the fermentative degradation of tissue leads in time to its isolation from contact with normal cells, and can in this fashion perform a certain protective function even despite the incomplete character of phagocytosis

The protective role of incomplete phagocytosis must not be overestimated in the light of the experimental data. On the basis of experiments with mobilized leucocytic exudate in the peritoneal cavity of white mice, a considerable protective effect was observed with various bacterial agents.

At the same time the investigation of mice resistant to such viruses which produce fatal infections after intraperitoneal inoculation (agents of tick-borne and Japanese encephalitis, equine encephalomyelitis viruses of WEE and EEE types) did not reveal any greater resistance of animals with mobilized leucocytic exudate as compared to normal mice. This was observed by means of quantitative titration of the LD_{50} of

TABLE VII
PHAGOCYTIC ACTIVITY OF LEUCOCYTIC EXUDATE OF WHITE MICE IN RESPECT TO DIFFERENT VIRUSES

Viruses	Virus-containing material[a]	Destruction of virus in leucocytes[b]
Tick-borne encephalitis	Brain of infected mice	None
Japanese encephalitis	Brain of infected mice	None
Poliomyelitis	Twenty-four hour tissue culture	None
Lymphocytic choriomeningitis	Brain of infected mice	Virus multiplies
Influenza	Chick erythrocytes	None
Rabies	Brain of infected mice	None
Epidemic parotitis	Infected amniotic membrane of living embryos	None
Ectromelia	Erythrocytes of humans and pigeons	None
Smallpox-vaccinia	Erythrocytes of humans and pigeons	None
Meningopneumonia of mice	Lung of infected mice	Yes
Ornithosis	Lung of infected mice	Yes

[a] Introduced into peritoneal cavity in form of particles 3–7 μ
[b] During period of complete tissue phagocytosis

the virus in the form of a purified suspension, and also in the form of a homogenized suspension of tissue particles from infected brain. The higher activity of elimination of the virus from susceptible cells, already referred to, is also observed with leucocytes, which are not able to isolate completely and retain for any length of time the virus particles attacked.

The inertness of phagocytic factors of protection in respect to the greater mass of viral agents is also evident from the study of the development of virus infections in animals with complete or partial block of the reticuloendothelial (RE) system (intravenous administration of India ink, iron saccharate, and also the removal of the spleen). In contrast to bacterial infections, which respond to blockage of the RE system in the form of increased severity of the experimental process, virus infections, as, for example, the experimental influenza of white mice studied by us in detail (Smorodintsev and Shishkina, 1940, 1942), pro-

ceed without any kind of deviation from the controls in respect to the dynamics of multiplication of the virus or of the mortality rate.

The barrier activity of the lymphatic nodes toward the bacterial agents is greatly enhanced in immunized animals, but is not changed toward viruses. When viruses are administered in sublethal doses into susceptible tissue of animals with active immunity, rapid virus destruction is observed and the organism is rapidly purged of virus Histopathological investigation of the process of purging of the susceptible organs provides most convincing confirmation of the role of phagocytic factors in antiviral immunity It is well known that inflammatory reactions induced by the exudation of phagocytic elements enter into the phenomena of interaction between bacterial agents and the tissues of immunized animals The introduction of massive sublethal doses of pneumococcus into the lung of white mice or guinea pigs with active immunity is followed by a rapid mobilization of the inflammatory reaction; the tissues are filled with phagocytic cells, which attack and destroy the pneumococci which have been sensitized by antibodies.

A totally different picture emerges when sublethal doses of influenza virus are introduced into the lungs of white mice or rabbits with active immunity The lungs are rapidly cleared of massive doses of influenza virus without any inflammatory phagocytic reaction: the viruses are rapidly blocked by specific antibodies in which they are bathed, thus leaving them out of contact with the mucous epithelium of the tissue, bronchi, and bronchioles, and causing them to die rapidly as a result of thermal denaturation In this way the organism rids itself of the equivalent of hundreds of thousands of normal lethal doses of virus virulent to normal mice, without using phagocytic processes, and without involving the processes of degeneration and necrosis of well-protected susceptible tissues For this reason our results indicate that histopathological analysis of the processes of purging of susceptible tissues of animals with active immunity is of the utmost importance in providing a quick answer to the question whether phagocytic factors play any part in protecting the organism against the agent under study If the immune organisms are able to cope with the problem of ridding the system of the agent completely without the intervention of phagocytic reactions, then these are clearly superfluous in the particular infection.

The method of histopathological analysis confirms the data presented earlier on the basis of more complex experimental investigations on the role of phagocytosis, and demonstrates the inactivity of phagocytic defense factors in all virus infections, with the exception of the psittacosis-

mouse pneumonia group, which also stimulate the mechanism of complete phagocytosis as part of the defense reaction.

VII. Effect of Nervous Regulation on Resistance to Virus Infection

The multiplication of viruses proceeds most favorably in organisms which are at the height of their physiological function, when the metabolism of the cells and tissues susceptible to the agent is at the optimum level. It is well known from experience of the production of live vaccines against smallpox and rabies that any lessening of the general resistance of the organism under the influence of cold, intoxication, trauma from burns or surgery, or concurrent bacterial infection, not only fails to strengthen the reproduction of the virus (as would be observed for bacterial agents), but regularly depresses the accumulation of virus and thereby depresses the quality of the smallpox or rabies vaccine

The relationship described does not, of course, imply a more benign course or a disappearance of the virus infection in the organism when the general resistance is depressed The slowing of the virus reproduction observed is only one aspect of the infective process, which is generally reflected in a greater vulnerability of the weakened organism to the action of toxic products of the virus itself, and to other additional factors such as concomitant bacterial infection The outcome of the virus infection can vary greatly, depending on the rate of multiplication of the virus in the sensitive tissues, the reaction of the latter to the virus proteins, the decomposition products of the infected organs, and the reversibility of the local and systemic damage caused by the virus The outcome and severity of the virus infection depends on two basic factors:

(1) The aggressive peculiarities of the agent, which is linked to its rate of multiplication in susceptible tissue, the degree of disorganization of host cell metabolism produced by the virus, and the concentration of the toxic substances produced by the virus

(2) The state of the protective reactions of the macroorganism in relation to the internal destructive reactions evoked by the virus These protective reactions are related to: (a) The continued intracellular virus destruction under the influence of nonspecific factors (thermal denaturation, thermolabile blood substances); (b) adaptive changes in the more important physiological functions (metabolism, respiration, cardiovascular system) suited to the changed conditions of existence of the organism; and (c) specific protective factors, coming into play gradually with the spread of the infective process

In the light of the physiological concept of Pavlov on the coadaptation of normal and pathological processes in the organism under the regulating influence of the nervous system, it seems essential to study the role of nervous regulation in the development of virus infections.

Action on the functional conditions of the central nervous system can result in profound metabolic changes in the organism, and in this context it can lead to alterations in the character of virus infections, leading to more benign or more severe courses.

In elaborating this work we have concentrated on the strictly parasitic functions of the viral agents, arising from a process of adaptation over a prolonged period, which convert them into highly active intracellular parasites

Even under the most unfavorable conditions for survival in the susceptible tissue, the viral parasite is capable of intracellular reproduction, with the maintenance of the specific genetic properties. The rate of reproduction of one and the same virus alters but little even in the presence of significant body temperature variations, while it is appreciably affected by factors which produce a significant lowering in the level of general resistance and a significant lowering of the level of metabolism (starvation, trauma arising from burns or surgery, intoxication, narcosis, etc).

Experimental action on the nervous system, leading to changes in the level of general reactivity of the organism, generally did not alter the basic biological properties of the parasite, but resulted in marked effects on the development of toxic or allergic processes, and on the development of specific immunological processes

In experiments in our laboratory (Smorodintsev, 1953, 1955), an examination was made of a variety of actions on the nervous system, producing changes in the nervous regulation of susceptible tissues, and capable of influencing their metabolic conditions.

Into this category falls the interference produced by surgical denervation of the lungs of white mice, and of the skin of rabbits, resulting in partial or complete isolation of the peripheral nerve endings.

We have followed the development of influenza virus in the lungs of white mice after preliminary crushing of the upper neck sympathetic nodes, after right-sided vagotomy, and bilateral section of the sympathetic nerve of the neck In other studies we have examined the development of smallpox-vaccinia virus in the skin of rabbits following partial or complete denervation of a dermal segment.

We also studied the development of influenza virus infection in the lungs of white mice after temporary isolation of the peripheral nerve receptors of the lung with Novocaine, atropine, sympatholytin. and also

under conditions of CNS depression produced by medication, as well as CNS stimulation with various pharmacological agents.

A. Development of Viral Agents in Denervated Tissue

Zhumatov (1955a) studied in our laboratory the multiplication of smallpox-vaccinia virus in the skin of rabbits, with various degrees of denervation but with intact blood supply The development of local inflammatory processes and temperature reactions was followed under these conditions in the denervated segment, in normal skin, and in the rectum A segment of tissue was prepared starting at the lower angle of the scapula, 9–10 cm in length, and 5–6 cm wide The segment retained its connection with the remaining skin through a narrow neck containing a main blood vessel with numerous collaterals All other blood vessels, together with the muscular layer, were separated from the underlying tissue by a sterile cellophane shield moistened with a solution of penicillin This shield protruded for a distance of 2 cm beyond the edge of the segment, and was sutured to the surrounding skin together with the skin segment In order to complete the denervation, Novocaine was introduced into the tissue proximal to the connecting portion and the walls of the main vessel were moistened with 5% phenol solution The isolated skin segment showed a pronounced lowering of response to galvanic stimulation after 4 days or more following the operation, when the rabbits were infected with vaccinia virus Histological examination of the segment after 4–5 days showed no intact nerve fibers following section At that time the protoplasm and nuclei of the epithelium still maintained their normal structure, while the temperature of the segment was regularly lowered by 2°–2½°C Experiments with 74 rabbits showed that the denervated skin had fully retained its sensitivity toward vaccinia virus, and that the rate of virus reproduction and its quantitative presence do not differ from that in normal tissue

Seventy-two hours after intradermal introduction of various dilutions of virus, the local inflammatory manifestations were less than in normal skin, and they disappeared more quickly Even considerable concentrations of virus did not produce vaccinia necrosis in the denervated segment The inflammatory process in the segment developed somewhat less intensely in rabbits, in which the exteriorized vascular trunk was treated with 5% phenol solution over a length of 1 cm in the tissue stem, which shows the transmission of a reflector impulse to the functioning organ In smears from the pustules of the denervated segment, elementary bodies were present in quantities usual for normal skin

Control experiments with hemolytic streptococci introduced in decreasing doses into the denervated tissue segment showed a pronounced

intensification of local microbial processes as compared to normal skin. In parallel with the intensified microbial reaction, there was an increased inflammatory reaction, and necrosis was produced in denervated skin with doses of streptococci which were inactive in normal skin. Such pronounced differences in the results with denervated tissue infected with smallpox-vaccinia virus and with hemolytic streptococcus are in our opinion explained by differences in pathogenesis and immunity as between viral and bacterial infections.

Skin denervation and vascular damage produced during the operation lead to a pronounced depression of phagocytosis and resorption of toxic microbial products This intensifies the multiplication of hemolytic streptococci and the severity of the pathological reaction.

The survival of the vaccinia virus depends primarily on the metabolic state of the epithelial cells The virus fails to react in any way to the changed phagocytic processes and the retarded resorption of the toxic substances can even have an unfavorable effect on its reproduction in the affected cells

In the denervated segment, the epithelial cells retain a fairly active metabolism Consequently the multiplication of virus is scarcely less than in normal skin.

Sokolovskaya (1953) has studied the development of experimental influenza infection in the lungs of white mice, after bilateral crushing of the upper sympathetic nodes of the neck supplying the lung tissue, under sodium amytal narcosis According to the data of Sokolovskaya and Monastirskaya (1953), this operation does not produce pneumonia, but leads to extensive destruction of vascular circulation in the form of vascular hyperemia with temporary atelectasis of lung tissue. The lung changes produced by this operation are not accompanied by the development of spontaneous bacterial pneumonia. Bacteriological examination of the lungs reveals a scanty and varied microflora.

The multiplication of influenza virus in the lungs of white mice following crushing of the upper sympathetic nodes of the neck proceeded without any noticeable change compared with the control group of animals

Only a slight depression of virus reproduction was observed in the experimental group without any effect on the mortality of the mice, and the virus dynamics in the group following operation did not differ from that of the control group.

Kurashvili (1955b) examined the influence of the functional state of the neuroreceptor apparatus of the mucous membranes of the upper respiratory tract and of the lungs of white mice on the reproduction of

influenza virus. The nerve receptors of the lungs were isolated temporarily or permanently by surgical interference (unilateral vagotomy, section of the sympathetic chain, crushing of the upper sympathetic ganglia of the neck) or with the help of pharmacological substances (intranasal introduction or inhalation of 0 1, 0 25, and 0 5% solutions of Novocaine, vagosympathetic block of the neck with Novocaine, atropinization of animals, introduction of sympatholytin), with retention of normal function of the CNS. As is well known, the isolation of the neural receptors produces changes in the physicochemical status of the tissues innervated by the receptors, and this could conceivably reflect on the multiplication of virus in a given tissue However, in these experiments it was not possible to detect any effect resulting from the isolation of neural receptors on the reproduction of influenza virus in the lungs of white mice These data do not minimize the role of the neurodystrophic component in the pathogenesis of bacterial infections, and relate merely to the nature of virus infections

B Effect of Depression and Stimulation of the CNS on Development of Virus Infection

The investigations of pharmacologists and biochemists have revealed considerable biochemical shifts and functional changes of individual systems and of the organism in general resulting from deep drug-induced sleep Into this category falls the disorganization of temperature regulation (lowering of body temperature), of blood pressure, of respiratory exchange (diminished oxygen requirements), and secretory and motor functions of the gastrointestinal tract (depression of these functions) A change in the number of erythrocytes and in their hemoglobin content is noted (especially when barbiturate derivatives are used) One of the principal protective mechanisms of the organism, phagocytosis, is suppressed The number of leucocytes is increased, but phagocytosis is depressed, the colloid-osmotic pressure of the serum falls, the sedimentation rate of erythrocytes diminishes, the blood coagulation is increased, the general protein content diminishes, the blood ammonia is markedly increased, the urinary excretion is significantly lowered, the concentration of hydrogen ions in the blood increases (acidosis), there are changes in the content of blood phosphate, potassium, sodium, and blood enzymes The contents of adrenaline, thyroidin, and of other hormones in the organism change

The development of the virus as an intracellular parasite depends on the metabolic status of the susceptible tissue which in turn depends on

the general status of the whole organism. A change in the supply of tissue nourishment or a change of substances in the organism will cause a disturbance in the metabolism of susceptible tissues, which of course will reflect on the fixation and reproduction of the virus.

All the above changes in the metabolism of the tissue of the trachea and bronchi of the lungs arise from a disturbance of the normal trophic function of the CNS, and these changes affect the activity of the whole organism.

This list of changes, by no means complete, occurs in the organism during narcosis and drug-induced sleep These changes vary with the different narcotics and soporifics in respect to their intensity and the localization of the various manifestations. Unfortunately, up to the present time the study of the mechanisms of intracellular metabolic processes and the nature of the specific action of viruses on them has been very inadequate.

The ability of the organism to react to a variety of stimuli is either diminished or totally abolished in narcotic depression of the CNS The possibility of pathological reflexes is limited or completely suppressed On removal of the narcotic depression of the CNS, the organism recovers its ability to react to the various stimuli

We first studied those narcotics which produced prolonged sleep in white mice with the minimum of intoxication. Among the various preparations (Sodium Amytal, Veronal, urethan, Hexonal, Luminal, Medinal, hexobarbitone, Pentothal) the best results in respect to duration and depth of sleep and safety for white mice was obtained with *Veronal* narcosis, obtained by rectal administration of a 0 75–1.75% solution, occasionally augmented by addition of Veronal to the diet Veronal proved to have little toxicity for mice (Kurashvili, 1953).

Soon after rectal administration of Veronal, symptoms of stimulation were evident for a brief period, with quickening of pulse and respiration, and development of clonic and tonic contractions. After 10–12 minutes *the mice fell into a deep, quiet sleep for a period of 14–18* hours. The sleeping mice did not react to external stimulation, the respiration slowed to 16–24 per minute, the pulse rate fell, the body temperature dropped 5–6°C, and the thermoregulation was greatly disturbed. The administration of Veronal was repeated after 4–6 days. During sleep the animals were kept under a thin layer of cotton wool, and their posture was changed periodically in order to prevent congestion in internal organs. The mice were awake for a period of 5–6 hours in the day during which time they fed themselves. This regimen of narcotic sleep resulted in a markedly lower mortality than continuous harmful nonphysiological sleep necessitating artificial feeding of the animals. Low-

ering of the daily dose of Veronal to 0.2 ml of 1.5% solution rectally per day per mouse, with frequent inspection of the sleeping mice, made it possible to lower the mortality from intoxication to a minimal figure (0–15%) in the control group.

The investigations of Kurashvili (1953) showed that depression of the CNS produced by Veronal narcosis sharply reduced the reproduction of influenza virus in the lungs of white mice. The infective dose of the virus used is critical in showing this effect, as is the depth and duration of narcosis and the length of the period during which sleep was repeatedly induced. As the infective dose of virus used was increased, the effect of the drug narcosis becomes less pronounced. A pronounced retardation of virus reproduction was only shown by those mice which had been narcotized for 3 days for a period of 12–16 hours daily. If the Veronal narcosis was started earlier, a more pronounced depression of the influenza virus was observed in the lungs of the mice. Veronal narcosis for a period of 3 days did not prevent death of the animals, but only prolonged their life for 2–3 days.

Protracted administration of Veronal narcosis (for a period of 10 days) and infection of the mice with small doses of influenza virus produced some therapeutic effect: the survival rate in the experimental group was 56–61% and in the control group 30–44%. In further experiments it was seen that this effect is not consistent and cannot always be obtained in the experiments. The experimental results depend on the age of the mice, their general condition, their nutritional state, and on the batch of Veronal used. The reproduction of the influenza virus in the lung was also markedly depressed in rats under Veronal narcosis.

A pronounced depression of the reproduction of the virus during the period of narcosis does not lead to its disappearance from the lung tissue. After termination of the narcosis rapid multiplication of the virus ensues, resulting invariably in the death of the mice, occurring after only a slightly longer period than in the controls; this is best observed when the mice are infected with a relatively small dose of virus (1–2 LD_{50}).

In analyzing the causes of the depressed virus development in narcotized mice, we have focused attention in the first instance on the possibility of a drop in body temperature in animals under Veronal narcosis. One could suppose that a lowering of the body temperature by 6°–8°C in sleeping mice could retard the multiplication of the virus as an intracellular parasite, since it requires optimal metabolic tissue conditions, which exist at the normal temperature of the organism.

However, Kurashvili (1953) has shown that the reproduction of virus in the lungs of hypothermic mice does not differ from that of the control animals. In fact, the hypothermic mice died somewhat more rapidly

than the controls. One can therefore state that the lowering of the body temperature in the narcotized mice cannot interfere with the reproduction of the influenza virus and cannot be the cause of retarded development of viruses in narcotic sleep.

As emerged later, the first stage of the interaction of the virus with the susceptible tissue—fixation of virus on the cell—proceeds more sluggishly in narcotized mice than in the controls, and in this respect it approaches the fixation of virus in immune animals. It also became apparent that the elution of influenza virus from the susceptible cell is somewhat increased in narcotized animals. All these factors can lead to a depression of the reproduction of influenza virus in narcotized mice

It is known that Veronal has a definite toxic effect. It was important to elucidate whether Veronal has a toxic action on the metabolism of tissues susceptible to the virus by a direct interaction of the Veronal with the cells of such tissues. It was shown that intranasal administration or inhalation of Veronal did not affect the course of influenza infection in mice and did not influence the rate of reproduction of influenza virus In this connection the depression of the reproduction of influenza virus in Veronal narcosis is more correctly explained in terms of a direct toxic effect on the metabolism of tissue susceptible to the virus, a blocking of the cells, or an action on the neural receptors of the lungs

The reproduction of virus in the lungs of alcoholized mice (0 4 ml. of 15% ethyl alcohol per oz) was depressed during the first 2–3 days after infection with doses of virus, but from the third day onward the virus multiplied without hindrance and reached the same concentrations as in the control animals Prolonged administration of alcohol (over a period of 10 days) aggravated the course of influenza infection in white mice (Kurashvili, 1955c) Alcohol intoxication of white mice significantly lowered their resistance to virus infection.

The general relationships observed in influenza infections of white mice under Veronal narcosis were also observed in experimental neuroviral infections. Drobyshevskaya (1955) infected mice with sublethal subcutaneous doses of tick-borne or Japanese encephalitis and observed the prolonged action of Veronal introduced per rectum as 1.75% solution once daily. After 3, 6, 9, and 12 days a quantitative virus titration was performed on the brain of individual narcotized and control animals It was *shown that during the first days of the experiment* the amount of virus in the brain of narcotized mice was significantly below that of the control animals After 3–6 days following the administration of the Veronal, the rate of multiplication of the virus was increased in the sleeping mice

In the group of animals subjected to the action of narcotic substances the mortality was 100%, with an average duration of survival of 8 3 days, while the mortality rate in the control group was 67% with an average duration of survival of 11 6 days

Hexenal had analogous effects on the multiplication of Japanese encephalitis virus in the brain of mice, when administered as 0 1% solution subcutaneously twice daily for a period of 4 days Up to the moment of termination of hexenal narcosis (after 4 days) a significant depression of the rate of virus development was observed in the sleeping mice, the virus was not evident in 10-fold dilutions, while in the control mice it was detectable in 1000-fold dilution Four days after termination of the narcosis the differences had been evened out, and the virus content was the same in the experimental and in the control groups. In these experiments also the mortality was rather higher in the narcotized animals In this case the average period of survival was longer in the narcotized group than in animals which had received a single dose of narcotic

In these experiments the retardation of virus development has been a regular feature with a variety of viruses in the susceptible tissues of narcotized animals, it is only observed with sufficiently deep and prolonged narcosis The considerable derangement of metabolic processes which always accompanies narcosis has evidently been the cause of the retarded virus development in the organism of narcotized animals, but the therapeutic effect of such "heroic" interference is at best doubtful and more often negative If either the depth or duration of narcosis are lessened the activity of virus reproduction is rapidly intensified This is even more pronounced when narcosis is terminated, when the viral process quickly reaches the same intensity as in the control animals

The correctness of our interpretation in ascribing the observed retardation of virus reproduction in the organism of narcotized animals to the profound interference of narcosis with the metabolic processes was confirmed in further control experiments, in which analogous results were obtained under the influence of starvation, burn trauma, and various intoxications in mice that were awake.

According to Pavlov (1938), all nervous activity is composed of sequential processes of depression and stimulation We have already discussed the development of influenzal infection in consequence of depression of the CNS in white mice In a series of other experiments the processes of depression were contrasted with those of stimulation of the central and vegetative nervous systems, and the development of experimental influenzal infection was studied in its light It is known

that stimulation of the CNS has an effect on all functions of the organisms, heightening the activity of all physiological systems. It thus also intensifies the metabolic processes in the organism.

It would seem, therefore, that as a result of the raised metabolic level the influenza virus as an obligatory intracellular parasite would multiply more rapidly. However, the administration of caffeine, phenamine, ephedrine, morphine, adrenaline, or acetylcholine produced no such effect: the influenza virus multiplied in the lung of white mice under stimulation at the same rate as in the (normal) control animals, while the course and the end result of the infection was more severe in the greater part of the animals (Kurashvili, 1955a).

The normal metabolizing cell is thus to be regarded as the optimum medium for virus reproduction Raising the metabolic level above its normal value, as the experiments show, does not intensify the virus reproduction, but increases the severity of the infective process. What is the cause for the less benign course of influenzal infection in mice subjected to stimulation of the CNS? The reasons must be found in differences in response reactions of the organism produced by different states of the CNS on a given concentration of virus in the organism.

Depression of the CNS lowers, and stimulation of the CNS raises the sensitivity of the organism to the virus and its toxin. For this reason an amount of virus, permitting survival in the normal animal, may yet be fatal to animals with stimulation of the CNS. All this evidence testifies to the effect of the functional state of the CNS on the development and the outcome of the infective process.

The function of the nervous system in the organism's fight against disease is thus not a narrow and circumscribed one in the sense of a direct action of the nervous system on the pathogenic agent and its destruction. This, of course, would be impossible. A pathogenic agent, in the case stated the virus, entering the susceptible cell unbalances the internal equilibrium of the organism through its specific action. In order to restore the equilibrium, the CNS mobilizes the physiological mechanisms in its arsenal, and directs them toward re-establishment of the internal milieu of the organism. With the re-establishment of the internal milieu, recovery ensues.

In the fight against virus infections, even a temporary interruption of the normal activity of the CNS is undesirable, as it leads to the disorganization of protective functions of the organism, and of the important immunological mechanisms

The observations described above show that only the most intense action on the CNS (such as deep narcosis), producing a depression or

interruption of many vital function of the organism, has any effect on the development of viral processes Under such conditions a less active multiplication of the virus is observed in the susceptible tissues, which however does not compensate for the harm done by the highly toxic action on the nervous system

In our experiments such extreme indexes of the action on the nervous system as death or survival of the animal, and multiplication or suppression of the virus in the susceptible tissues were used A substantial defect of the action employed on the nervous system was its relatively high intensity.

It is possible that the use of small doses of narcotics could facilitate the production of a therapeutic (medicinal) sleep, not having any effect on the fate of the infected organism, but exerting a beneficial effect on isolated aspects of virus infection, such as diminishing the virus toxicosis of the nervous system, and relatively minor disorganization of the more vulnerable forms of its activity, especially the conditioned reflex activity

In our joint studies with D A Biryukov, it was established that the conditioned reflex activity of white mice infected with influenza virus was profoundly disorganized, even during a late stage of convalescence, long after the virus had disappeared from the organism (Smorodinstev, 1955). It is quite evident that under such conditions gentle medicinal sleep has a beneficial effect on the preservation and rapid recovery of the conditioned reflex activity of the cortex of the cerebral hemispheres, which can speed the recovery from illness in man

Thus the development of viral agents in the organisms of susceptible experimental animals reflects the parasitic type of process firmly established in the evolutionary scale, retaining a high intensity even despite significant disorganization of the normal physiological activity of the susceptible tissue

In this context even drastic interference with the nervous system associated with denervation of susceptible tissue, or the administration of large doses of pharmacological narcotics or stimulants, etc, does not seem capable of interrupting the normal course of reproduction of the agent, or of influencing the outcome of the virus infection

Only the prolonged narcosis depressed significantly the reproduction of the influenza neuroviruses studied, which must be explained in terms of the profound disorganization of metabolism in the susceptible tissues Termination of the narcosis, or a lowering of the narcotic dosage, quickly removes the differences in the rate of reproduction of viruses in comparison with normal animals

The weakening of pathological reactions during development of the

virus in denervated tissue does not signify any lessening of severity of the infectious process but reflects a weakening of the responsible protective reactions on the part of the organism.

It is very important to study further the most important mechanisms of nonspecific resistance to virus infection in animals and man.

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